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ENZYMES IN BLOOD

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ENZYMES IN BLOOD*

Conference Chairman and Consulting Editor
LAURENS P. WHITE

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* This series of papers is the result of a conference on *Enzymes in Blood*, held by The New York Academy of Sciences on February 26 and 27, 1958.

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INTRODUCTION

By Laurens P. White
Stanford University School of Medicine, San Francisco, Calif.

In the past ten years there has been an explosive increase in interest in the enzymes of blood, both in serum and in cells. Many laboratories have become concerned with one or another aspect of this intriguing field, and considerable bodies of data have been accumulated. Because of the differences in the type of material within blood such studies have been confined, necessarily, either to leukocytes, erythrocytes, or to serum, generally without regard for the other constituents of blood.

Enzymologists have long regarded blood as a contaminant of their tissue preparations and cursed it rather than assayed it. Clinicians, impressed by the ease of frequent, adequate sampling of blood and the elegance of spectrophotometric enzyme assay, have done a great deal of work in the investigation of enzyme alterations in the cellular and serum compartments of blood. These studies have been pursued in every imaginable illness, and many different enzymes have been measured. In only a few cases, chiefly with cellular enzymes, have such clinical studies been directed toward an understanding of the mechanism of enzyme alteration in disease. Factors such as the movement of enzymes across cell membranes, the origin and excretion of enzymes of serum, and the characteristics of the enzyme under study have largely been ignored in these clinical studies.

At the same time, enzymologists and physical chemists, whose training and interest equip them well for such basic studies, have turned their attention to blood and have begun studies of immediate significance in this field. It is, however, a sad reality that enzymologists and clinical investigators do not usually communicate with each other at any level more basic than that of casual social intercourse.

The conference on which this monograph is based was therefore planned with the specific goal of increasing the contact between clinical investigators of blood enzymes and members of other groups, such as enzymologists and physical chemists, so that the skills of each, the problems of each, and the deficiencies of each might, at one point in time, be viewed by all and that, through such an appraisal, each might gain in understanding and some increased ability for his own investigations.

Part I. Enzymes in White Blood Cells

LEUKOCYTE GLYCOLYSIS: AN INVESTIGATION OF THE FACTORS CONTROLLING THE RATE BEHAVIOR IN MULTIENZYME SYSTEMS*

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Introduction

The fact that the investigation of the metabolism and enzymology of human leukocytes has flourished in recent years is reflected in the several conferences and seminar series that have been held on this subject since 1952. The published proceedings of some of these meetings[1-3] give ample evidence of the increasing vigor and promise of this field of inquiry. It appears that interest in this field is increasing and that a scattering of pioneering investigators has begun to study the biochemistry of those cells that occasionally contaminate our leukocyte preparations: the erythrocytes and the platelets.

Despite the recent surge of interest in the leukocyte, the fact is that leukocyte metabolism has been under investigation for well over fifty years. The early classic demonstration that lactic acid was derived from glucose and not from protein metabolism was made on leukocyte-rich exudate material.[4, 5] In the intervening years a great many observations were made on the glycolysis, proteolytic activity, histochemistry, chemical composition, and miscellaneous enzymology of leukocyte preparations of diverse origin and, although many of these investigations suffered from inadequacies of the isolation methods and other techniques, it became increasingly clear that the blood leukocyte is an exceedingly interesting cell whose remarkable chemistry, biology, and pathology make it a tantalizing object for investigation.

With the possible exception of mouse ascites tumor cells, leukocytes are probably the only free-floating nucleated cells that can be isolated easily from mammalian sources. They can be accurately enumerated and are perhaps ideally suited for study under simulated *in vivo* conditions. Unlike ascites tumor cells, malignant leukocytes have clearly defined normal analogues that may serve as useful controls for metabolic investigation. Also ascites tumor cell suspensions are often contaminated with leukocytes, but suspensions of blood leukocytes are almost never contaminated with mouse ascites tumor cells. Finally, leukocytes have a number of fundamental biological properties, such as phagocytosis and motility, with which one may seek to correlate observed biochemical properties, a fact that greatly increases their usefulness and interest.

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† Established Investigator of the American Heart Association.

My own interest in leukocyte glycolysis began after the observation had been made[6, 7] that isolated blood leukocytes from patients with chronic myelocytic leukemia and chronic lymphocytic leukemai have considerably lower levels of alkaline phosphatase activity per cell than do normal leukocytes. Conversely, the leukocytes of leukocytosis and a variety of myeloproliferative disorders (for example, myeloid metaplasia and polycythemia vera with leukemoid features) all had higher-than-normal levels of alkaline phosphatase activity. Since the striking decrease of phosphatase activity in myelocytic leukemia was a consistent finding that persisted throughout therapeutically induced remissions and, in a number of cases, was detected in advance of the appearance of clinical leukemia,[8, 9] an investigation was begun on the possible metabolic significance of such alterations. The following discussion is concerned only with observations made on normals, chronic myelocytic leukemia, and chronic lymphocytic leukemia leukocytes.

As a first approach, measurements were made of the aerobic glycolytic rates of normal and leukemic leukocyte homogenates. Leukocytes were isolated by the bovine fibrinogen method[10] and were washed and homogenized by procedures that have been described elsewhere.[11, 12] Lactic acid production, oxygen utilization, and glucose utilization were measured after incubation in air at 37°C. in a system containing glucose (0.005 M), potassium phosphate buffer, *p*H 7.4, (0.01 M), MgCl$_2$ (0.005 M), adenosine triphosphate (ATP) (0.001 M), diphosphopyridine nucleotide (DPN) (0.0007 M), cytochrome *c* (1.4×10^{-5} M), and aliquots of leukocyte homogenate containing the equivalent of 2 to 5×10^7 cells.

The results of this survey are shown in TABLE 1. One can see that the metabolisms of the three cell populations studied were all primarily glycolytic, and that both leukemic cell types had rates of lactic acid production that were substantially lower than normal. It had long been known that human leukocytes have high aerobic glycolytic rates; in fact, this observation in normal cells was an important source of difficulty for the Warburg school which, in the late 1920s and 1930s, held that a high aerobic glycolytic rate was the *sine qua non* of malignant growth.[13] To resolve the dilemma posed by the

TABLE 1

AEROBIC LACTIC ACID PRODUCTION, OXYGEN CONSUMPTION AND GLUCOSE UTILIZATION IN LEUKOCYTE HOMOGENATES

The incubation mixture is described in the text. The figures are means of more than 60 determinations in each case. Results are expressed in μmoles per 10^8 cells per hour.

Observation	Type of leukocyte		
	Normal	Myelocytic leukemia	Lymphocytic leukemia
Lactic acid production	45.2	13.2	4.8
Oxygen consumption	4.0	1.2	1.1
Glucose utilization	21.0	5.8	2.9
Lactic acid production* / Oxygen consumption	36.8	33.0	13.1

* Ratio calculated after expressing both activities in terms of glucose equivalents.

TABLE 2

CONVERSION OF UNIFORMLY LABELED GLUCOSE-C^{14} TO LACTIC ACID AND CO_2 IN HOMOGENIZED AND INTACT LEUKOCYTES

Incubations contained the equivalent of 10^8 homogenized or intact cells, glucose-U-C^{14} (56,900 to 67,200 counts per minute), and the other additions usually present in the aerobic glycolysis system. Incubation, 120 minutes at 38°C. Figures represent changes in total radioactivity in counts per minute per incubation.

Compound	Type of leukocyte					
	Normal		Myelocytic leukemia		Lymphocytic leukemia	
	Homog.	Intact	Homog.	Intact	Homog.	Intact
Glucose	−30,800	−26,900	−10,200	−9,250	−7,280	−5,900
Lactic acid	+20,300	+23,300	+7,940	+8,080	+6,150	+5,150
CO_2	+980	+1,372	+435	+443	+188	+179
Radioactivity unaccounted for	9,250	2,228	1,825	727	942	571

finding of high aerobic glycolysis in certain normal cells, these workers took the position that this metabolic pattern might be seen also in injured cells. Since, it was stated, leukocytes could scarcely be handled without suffering injury, the unusual metabolism is thus explained.[14] However, adequate independent evidence of injury was not presented and, as has already been noted,[15] this view did not withstand later scrutiny. The fact that in this case the malignant analogue has a lower aerobic glycolytic rate than normal would seem to cast further doubt on the possible significance of this generalization, at least in the pattern of malignant growth we call leukemia.

In the experiment shown in TABLE 2, it is seen that in the glycolysis of uniformly labeled glucose-C^{14} (glucose-U-C^{14}) most of the disappearing glucose radioactivity appears in lactic acid. These results also confirm the quantitative differences among the three tissues and suggest that under these conditions there is little difference in glycolytic rate between homogenized and intact leukocytes.

The next question that arose was what explanation there might be for the observed differences in glycolytic rate between normal and leukemic leukocytes. It quickly became apparent that this was but a special case of the more general question: What controls the glycolytic rate of any tissue? This, of course, is but a special case of the even more general question: What controls the rate behavior of any metabolic multienzyme system? Throughout the huge literature on glycolysis, it is often stated that the glycolytic rate of a tissue is determined by a single rate-limiting step in the sequential chain of reactions, but the identity of this step is unknown. It is also unknown whether the rate-limiting step is the same in all tissues or whether it remains the same in pathological variants showing altered glycolytic rates.

A large literature has accumulated on the general problem of rate behavior in metabolic multienzyme systems. These writings have invariably emphasized the practical and theoretical difficulties inherent in any attempt to predict the rate behavior of a complex system from knowledge of its component parts. As complexity is increased by such factors as branching points, coupled reactions, fast steps, and catalyst regeneration cycles, these difficulties grow more formidable. Dixon, for example, in his stimulating essay on multienzyme systems,[16] wrote that "it is scarcely practicable to work out the complete kinetics of a chain of enzyme reactions quantitatively." Hearon[17] has nevertheless concluded on theoretical grounds that rate mastery may be assigned to a slow reversible step when certain conditions are met. Rate mastery by the first step represents the simplest case (in the presence of excess initial substrate) and mastery may be assigned to the first step when it is slow and irreversible or, if reversible, when the second step is fast.

In the light of these theoretical considerations and in view of the fact that few efforts have actually been made to analyze these problems systematically in single tissues, an attempt was launched to analyze this complex metabolic multienzyme system in sufficient detail to permit identification of the rate-limiting enzyme or enzymes whose activity levels or other properties make them pacemakers for the over-all system. Human leukocytes are particularly well suited to this type of study, both for their high aerobic glycolytic rates and for the fact that pathological variants are available that permit useful comparisons between the rate-controlling mechanisms of qualitatively similar multienzyme systems differing only in over-all rate.

Levels and Properties of Individual Glycolytic Enzymes

The approach to the problem of rate control consisted initially of systematic assays of a majority of the glycolytic enzymes in leukocyte homogenates.[18, 19] In TABLE 3, the results are given on a comparative basis, with the activities in normal preparations equal to 100. Comparable data are also given for the overall glycolytic rates. It is of interest to note that, of the enzymes assayed, the levels of all but four (phosphohexose isomerase, aldolase, triosphosphate isomerase, and pentosephosphate isomerase) were found to be roughly proportional to the levels of over-all glycolysis. These four are present in the same concentrations in myelocytic leukemia cells as in normal ones, despite the fact that glycolysis in leukemic cells is lower than normal.

In searching for prospective rate-limiting steps, the necessary comparison is that between the maximum capacities of the individual enzymes ($V_{max.}$) and and the maximum capacity of the over-all system. This information was obtained by computing the $V_{max.}$ for each enzyme in Lineweaver-Burk plots. The results show (TABLE 4) that, of the maximum capacities measured, that of hexokinase most closely correponds to the maximum capacity of the over-all glycolytic system, with phosphofructokinase a close second. Several enzymes (notably the ketose-aldose isomerases, phosphohexose, and phosphopentose isomerase) are present in enormously larger amounts than is apparently necessitated by their role in glycolysis. Interestingly, both dehydrogenases of the phosphogluconate oxidative pathway (glucose-6-phosphate and 6-phosphogluconate dehydrogenase) have lower $V_{max.}$ than that of the over-all glycolytic system, presumably placing an upper limit on the volume of metabolic traffic capable of entering this pathway.

TABLE 3

COMPARISONS OF THE AEROBIC GLYCOLYTIC RATE AND GLYCOLYTIC ENZYME ACTIVITIES OF NORMAL, MYELOCYTIC LEUKEMIA, AND LYMPHOCYTIC LEUKEMIA LEUKOCYTES

The figures represent activity levels relative to mean activity in normal cells (set equal to 100) ± standard error of the mean.* The data from which these ratios were calculated were expressed as activities per 10^{10} cells.† Each figure represents the mean of separate determinations on the leukocytes of 4 to 18 individuals. All but 3 of the figures represent the averages of 10 or more determinations.

Activity	Type of leukocyte		
	Normal	Myelocytic leukemia	Lymphocytic leukemia
Aerobic glycolysis	100 ± 12	29 ± 4	11 ± 2
Hexokinase‡	100 ± 12	47 ± 5	17 ± 2
Phosphohexose isomerase	100 ± 11	100 ± 13	26 ± 3
Phosphofructokinase‡	100 ± 15	42 ± 5	17 ± 2
Aldolase	100 ± 6	107 ± 5	35 ± 1
Triosephosphate isomerase	100 ± 11	106 ± 8	32 ± 3
Glyceraldehyde-3-phosphate dehydrogenase	100 ± 12	59 ± 6	19 ± 2
Pyruvate kinase	100 ± 13	37 ± 5	13 ± 3
Lactic dehydrogenase	100 ± 12	41 ± 3	24 ± 3
Glucose-6-phosphate dehydrogenase	100 ± 10	38 ± 4	18 ± 1
6-Phosphogluconate dehydrogenase	100 ± 12	41 ± 3	17 ± 1
Phosphopentose isomerase	100 ± 11	98 ± 12	24 ± 3

* Calculation of the standard error of the mean, s.e. = $\sqrt{\Sigma d^2/n\ (n - 1)}$.

† Six cell counts were done on saline suspensions prior to homogenization. When the standard deviation of the mean of the suspension counts was computed and expressed as a percentage, the mean standard deviation of a series of 187 suspensions was 8.1 per cent. The standard deviation of this mean was 2.1 per cent.

‡ A small number of determinations gave erratically low values and were discarded before calculating the mean.

To rule out the possibility that the apparent differences in enzyme levels between normal and leukemic tissues are, in fact, due to differences in the properties of normal and leukemic enzymes, conparisons were made between the Michaelis constants (K_m) and *p*H-activity curves of analogous normal and leukemic enzymes. No differences were noted in these parameters between the normal and leukemic forms of any of the enzymes studied, and it was found that the constants and *p*H behavior observed generally agreed with reported values for corresponding enzymes from other biological sources.

Role of Hexokinase and Adenosine Diphosphate

If hexokinase is the rate-limiting enzyme of glycolysis, an increase in the rate of lactic acid production would be expected if the level of hexokinase were increased by supplementation with pure enzyme or if hexokinase were bypassed by using glucose-6-phosphate (G-6-P) as substrate. Both predictions are borne out in normal leukocyte preparations by the data in TABLE 5. Doubling the hexokinase level by supplementation with purified yeast enzyme increases the hexokinase glycolytic rate almost 50 per cent and further additions of hexokinase increase it no further. A similar increase occurs with glycose

TABLE 4

COMPARISONS OF $V_{MAX.}$ AND K_M VALUES OF GLYCOLYTIC ENZYMES OF NORMAL AND LEUKEMIC LEUKOCYTES

Velocities expressed as μmoles of triose or triose equivalents produced or converted per minute per 10^{10} cells. For purposes of comparison, one mole of the substrates of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and phosphopentose isomerase is considered equivalent to 2 moles of triose.

Activity	Computed $V_{max.}$			K_s*	K_o*
	Normal	Myelocytic leukemia	Lymphocytic leukemia		
Aerobic glycolysis	75	22	8	2.5×10^{-4}	
Hexokinase	86	40	15	3.5×10^{-4}	3.0×10^{-4}
Phosphohexose isomerase	4950	4950	620	1.7×10^{-4}	
Phosphofructokinase	115	48	19	1.8×10^{-4}	2.5×10^{-4}
Aldolase	155	167	53	7.3×10^{-4}	
Triosephosphate isomerase	292	310	93	3.9×10^{-4}	
Glyceraldehyde-3-phosphate dehydrogenase	512	320	96	3.4×10^{-5}	4.1×10^{-5}
Pyruvate kinase	162	60	20	$1.0 \times 10^{\ 4}$	—
Lactic dehydrogenase	600	360	180	2.5×10^{-5}	4.9×10^{-5}
Glucose-6-phosphate dehydrogenase	55	21	10	1.7×10^{-5}	1.3×10^{-5}
6-Phosphogluconate dehydrogenase	45	18	8	1.2×10^{-5}	2.1×10^{-5}
Phosphopentose isomerase	4800	4690	1150	1.4×10^{-3}	

* Means of normal, myelocytic leukemia, and lymphocytic leukemia values.

TABLE 5

EFFECT OF SUPPLEMENTATION WITH PURIFIED ENZYMES ON RATES OF LACTIC ACID PRODUCTION IN GLUCOSE AND GLUCOSE-6-PHOSPHATE GLYCOLYSIS

The figures represent glycolytic rates relative to the rate (set equal to 1.00) in the usual aerobic glycolysis system with glucose as substrate, as described in the text. Purified hexokinase was added in amounts which yielded final hexokinase levels per flask of 2, 5, and 10 times the endogenous level, respectively. Other enzymes were added in amounts needed to increase endogenous activity fivefold. When used, the amount of added TPN was 0.3 μmoles. Substrate concentrations were 0.005 M. Incubation times, 2 hours.

Substrate	Other additions	Type of leukocyte		
		Normal	Myelocytic leukemia	Lymphocytic leukemia
Glucose		1.00	1.00	1.00
Glucose	Hexokinase (× 2)	1.46	1.81	
Glucose	Hexokinase (× 5)	1.39	3.04	1.96
Glucose	Hexokinase (× 10)	1.49	3.04	
Glucose	Glucose-6-phosphate dehydrogenase; TPN	1.03	1.08	0.97
Glucose	Aldolase	1.06	1.01	1.05
Glucose	Glyceraldehyde-3-phosphate dehydrogenase	1.02	1.06	
Glucose	Lactic dehydrogenase	1.09	1.09	
G-6-P		1.42	0.92	1.02
G-6-P	Glucose-6-phosphate dehydrogenase; TPN	1.58	1.15	1.10

TABLE 6

EFFECT OF ADDED ADP ON THE RATE OF LACTIC ACID PRODUCTION FROM VARIOUS SUBSTRATES

The figures represent glycolytic rates relative to the rate (set equal to 1.00) in the usual aerobic glycolysis system with glucose as substrate, as described in the text. ATP (4 μmoles) was present in all flasks. ADP (4 μmoles) was added to the flasks designated +. In flasks to which purified hexokinase was added the final hexokinase level per flask was 5 times the endogenous level. Incubation time was 2 hours.

Substrate	Other additions	ADP	Type of leukocyte		
			Normal	Myelocytic leukemia	Lymphocytic leukemia
Glucose		−	1.00	1.00	1.00
Glucose		+	1.29	2.25	2.16
Glucose	Hexokinase	−	1.61	3.04	2.78
Glucose	Hexokinase	+	1.86	3.46	3.15
HDP*		−	1.38	1.26	1.30
HDP		+	2.00	2.40	2.25
R-5-P*		−	0.19	0.42	0.22
R-5-P		+	0.37	0.67	0.49

* Concentration, 0.005 M.

6-phosphate as substrate. With myelocytic leukemia leukocytes, however, a 300 per cent increase occurs upon continued addition of hexokinase but, interestingly, no increase occurs with glucose-6-phosphate as initial substrate. The failure of glucose-6-phosphate glycolysis to exceed glucose glycolysis implies the critical necessity of active glucose phosphorylation as a source of adenosine diphosphate (ADP) and suggests that ADP production may be abnormally low in leukemic cells. Data supporting this conclusion are shown in TABLE 6. When ADP is added to glycolyzing systems containing glucose and the usual amount of added ATP, a larger per cent of increase in the rate of lactic acid production is observed in the leukemic cell homogenates than in the normal.

These results would be explained if leukemic cells were deficient in ADP generating systems. Experiments were done to test the effect of adenosine triphosphatase (ATPase) supplementation on the rate of lactic acid production and the time course of ketohexose and triosephosphate concentrations as measured by a modification of the cysteine-carbazole method of Dische and Borenfreund.[20] As has been reported in detail elsewhere,[18, 19] the results showed that the lactic acid production of ATPase-supplemented myelocytic leukemia leukocyte preparations was not stimulated by the addition of ADP as was the unsupplemented system. In addition, the steady-state concentrations of ketohexose and triosephosphate were lower in the ATPase-supplemented preparations, as were the levels of these intermediates in those preparations to which ADP had been added instead of ATPase. Similar though less clear-cut effects were observed when this experiment was repeated with lymphocytic leukemia leukocyte homogenate. The addition of similar amounts of ATPase to normal leukocyte homogenate had no apparent effect other than a slight increase in the rate of lactic acid production.

Supplementation with the other glycolytic enzymes tested (TABLE 5) had no effect on the glycolytic rate in normal and leukemic preparations. Fructose 1,6-diphosphate (HDP) glycolysis was somewhat higher than glucose glycolysis in all three tissues (TABLE 6). This may be related to the generally higher levels of the enzymes catalyzing the HDP → lactate sequence. However, the per cent effect of ADP addition in stimulating HDP glycolysis was greater in leukemic preparations than in normal. Although all of the enzymes of the HDP → lactate sequence have not been studied in detail, the data indicate that when adequate ADP is present the rate of lactic acid production from HDP is similar to the $V_{max.}$ of pyruvate kinase in all three tissues. This, then, may be the rate-limiting enzyme of the subsequence.

In studies now in progress (Hays and Beck, unpublished data) ATPase activity is being assayed directly. It has not been possible thus far to assess the role of ATPase in the physiological production of ADP deficiency in leukemic cells. The evidence has suggested that, although a phosphatase, ATPase is a distinctly separate activity from alkaline phosphatase, having a different *p*H optimum and a different distribution among the cell types of the peripheral blood. For example, it appears that, unlike alkaline phosphatase, ATPase is active in lymphocytes and eosinophils. Consequently, we have not achieved our original goal, which was to clarify the metabolic significance of the altered level of alkaline phosphatase activity in leukemic leukocytes.

Occurrence and Control of the Phosphogluconate Pathway

An attempt was also made to measure the extent of metabolism proceeding via the so-called hexose monophosphate shunt or phosphogluconate oxidative pathway in leukocytes. The question is of some interest, since the initial step in the shunt path forms a branching point in a larger sequence whose own pacemaker appears to be the step immediately preceding the branching point. This raises the possibility that the direction of metabolism may be controlled by the availability or concentration of the substrate, which faces alternative pathways at the branching point (that is, glucose-6-phosphate) rather than by the concentration of the individual enzymes.

Since it has already been shown that a major percentage of the utilized glucose is converted to lactic acid, it may be assumed that only a relatively small fraction traverses the oxidative pathway. This supposition was confirmed both by "positive" methods in which the production of $C^{14}O_2$ from glucose-1-C^{14} and glucose-U-C^{14} (or glucose-6-C^{14}) was compared, and by "negative" methods in which calculations were based on the relative specific activities of lactate-C^{14} formed from glucose-U-C^{14} and glucose-1-C^{14}. As shown in TABLE 7, somewhat less than 10 per cent of utilized glucose traverses the phosphogluconate pathway, although this percentage is higher in leukemic cells than in normal cells.[19]

In a series of experiments in which the production of fructose-6-phosphate and TPNH were both measured in a two-minute incubation with either glucose or glucose-6-phosphate as substrate, it was shown that the total amount of substrate entering the shunt pathway and the Embden-Meyerhof (E-M) glycolytic pathway and the fraction of this total entering the shunt pathway

TABLE 7

PERCENTAGE OF GLUCOSE CATABOLIZED VIA THE PHOSPHOGLUCONATE PATHWAY IN LEUKOCYTES

The figures represent percentage of glycolyzed glucose entering phosphogluconate pathway.

Calculation	Type of leukocyte					
	Normal		Myelocytic leukemia		Lymphocytic leukemia	
	Homog.	Intact	Homog.	Intact	Homog.	Intact
$C^{14}O_2$ method:						
from glucose-1-C^{14} data	2.1	2.2	3.8	3.7	3.3	3.1
from glucose-U-C^{14} data	3.7	3.6	8.0	8.2	9.8	9.7
Lactate-C^{14} method:	2.2	—	5.3	—	13.3	—

are both directly influenced by the concentration of available glucose-6-phosphate. When the only available glucose-6-phosphate was that produced from added glucose by tissue hexokinase, the amount of glucose-6-phosphate entering the shunt was less than 11 per cent of the total glucose-6-phosphate entering both pathways. As the glucose-6-phosphate concentration was increased, either indirectly by increasing hexokinase levels in glucose incubations or directly by adding glucose-6-phosphate in increasing amounts, there was a large increase in the amount of glucose-6-phosphate entering the E-M pathway and a corresponding decrease in the percentage entering the shunt.

This corresponds with the predictions that might have been made on the basis of the observed $V_{max.}$ and K_m values for the two enzymes, phosphohexose isomerase and glucose-6-phosphate dehydrogenase. It is concluded, therefore, that while an upper limit is set upon the amount of substrate entering the phosphogluconate pathway per unit time by the $V_{max.}$ of tissue glucose-6-phosphate dehydrogenase, the important operating factor in determining the actual amount entering the shunt, at least initially, is the existing glucose-6-phosphate concentration. In glucose glycolysis, this would be dependent upon the level of hexokinase activity and the concentration of added glucose in the presence of adequate ATP.

These observations suggest a possible explanation for the higher-than-normal percentages of shunt metabolism observed above in leukemic cells. If, in glucose glycolysis, glucose-6-phosphate were maintained in an abnormally low concentration, the percentage of utilized glucose-6-phosphate entering the shunt would increase. The most probable cause for such a depression in the steady state glucose-6-phosphate concentration would be a low level of hexokinase per cell in relation to phosphohexose isomerase activity. That this is the case in leukemic cells is suggested by the experiment shown in TABLE 8, wherein the effect of supplementary hexokinase on the percentage of glycolyzed glucose entering the shunt was observed. Added hexokinase invariably increased the *amount* of $C^{14}O_2$ produced from glucose-1-C^{14} but, in all cases, the *percentage* of glucose entering the shunt decreased. It is seen,

TABLE 8

EFFECT OF HEXOKINASE LEVELS ON PERCENTAGE OF UTILIZED GLUCOSE ENTERING PHOSPHOGLUCONATE PATHWAY

The table gives percentages of glycolyzed glucose entering the phosphogluconate pathway as determined from the rates of $C^{14}O_2$ production from glucose-6-C^{14} and glucose-1-C^{14}. The amounts of hexokinase added resulted in a fivefold increase in total hexokinase activity per flask.

Added hexokinase	Type of leukocyte		
	Normal	Myelocytic leukemia	Lymphocytic leukemia
Absent	2.1	3.8	3.3
Present	1.9	1.8	2.1

however, that the extent of the decrease was greater in leukemic cells than in normal ones.

Discussion

These results may be considered from several points of view. First, they bear upon the general problem of rate behavior in metabolic multienzyme systems. It is needless to point out that the solutions of many or most of the problems of physiology rest ultimately upon the solutions of appropriate rate problems. Surely this is true for the problems of drug and hormone action, for the understanding of the factors governing choices of alternative metabolic pathways, and for the increasingly better understood problems of genetically controlled enzyme deficiencies, whether the resulting mutants are complete or "leaky." The last area is well exemplified by the recent discovery of the enzymatic basis of erythrocyte sensitivity to primaquine,[21] which is discussed elsewhere in this publication. One may hope that in future years more and more quantitative studies will be undertaken on the rate behavior of other metabolic multienzyme systems. Only in this way will the qualitatively well-studied pathways of metabolism be given truly physiological significance.

As for leukocyte glycolysis, a statement cannot be made at this time about rate control in intact cells where permeability, diffusion, and structural orientation undoubtedly play a part. However, the present results have suggested that over-all glycolytic rates are the same in homogenates and intact cells; preliminary results indicate fundamentally similar concentration patterns of various intermediate pools. In any event, the data re-emphasize the point that the leukemic leukocyte does possess a characteristic and abnormal metabolic pattern.

Finally, returning to the subject of this monograph, we see that the normal and leukemic leukocyte carries the complement of glycolytic enzymes, and one might readily visualize how, in the leukemic state wherein massive quantities of leukocyte material are constantly turning over, increases may occur in serum levels of the various glycolytic enzymes. Thus, it has been shown[22] that in myelocytic leukemia, serum phosphohexose isomerase rises in proportion to the peripheral leukocyte count. As we have seen, leukocyte phosphohexose isomerase is exceedingly high relative to the other glycolytic enzymes; hence one might reasonably expect this enzyme to be among the first to reflect excessive leukocyte breakdown. The same might be said for phosphopentose isomerase. What use or significance these observations may have for us in our approach to the leukemia problem is, of course, conjectural. Perhaps the conference on which this monograph is based will point the way to an understanding of the problem.

Summary

Marked differences are observed in the aerobic glycolytic rates of normal, chronic myelocytic leukemia, and chronic lymphocytic leukemia leukocytes, whose respective rates of lactic acid production from glucose were 45.0, 13.2, and 4.8 μmoles per hour per 10^8 cells.

An attempt was made to explain these differences (and thus to examine also the more general question of rate behavior in multienzyme systems) by identifying the rate-limiting or master reaction in the three tissues. Studies were made of the activity levels and maximum capacities of the individual glycolytic enzymes, and comparisons were made, first, of the individual enzymes with each other, second, of the individual enzymes with the over-all glycolytic systems, third, between the time-course of the concentrations of various intermediates and their steady state concentrations as predicted from the kinetics and equilibrium constants of the component enzymes, and finally, of these patterns in normal tissues with those in leukemic tissues.

The results show that hexokinase is rate limiting in all three tissues. Since leukemic cells are hexokinase deficient, their glycolytic rates are diminished. In addition, hexokinase (together with phosphofructokinase and possibly other enzymes) is a significantly more important factor in maintaining an adequate ADP level in leukemic homogenates than in normal ones because the former are also deficient in other ADP generating systems. Thus, ADP formation in leukemic homogenates is doubly impaired and the glycolytic rate is correspondingly diminished. Similar mechanisms may operate in the intact cell.

The phosphogluconate oxidation pathway occurs in leukocytes, though less than 10 per cent of utilized glucose traverses this pathway. This percentage is higher in leukemic cells than in normal ones. The extent of alternative pathway metabolism is shown to be under direct control of the glucose-6-phosphate concentration and indirect control of the hexokinase level. The higher percentage of phosphogluconate pathway metabolism in leukemic cells is attributed to their deficiencies in hexokinase.

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Discussion of the Paper

Richard Wagner (*Tufts University School of Medicine, Boston, Mass.*): It would be interesting to know whether glycogen degradation was studied during the incubation period described by William Beck, who has demonstrated that there are considerable differences in hexokinase activity between normal and leukemic cells. It seems to me that differences in the rate of glycogen degradation are also to be expected, since free glucose inhibits the activity of phosphorylase.

ENZYME STUDIES ON WHITE BLOOD CELLS*

By Richard Wagner and Rose Sparaco
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Introduction

Enzyme studies on white blood cells are difficult to evaluate for many reasons, one of which is the problem of obtaining material free from contaminants, particularly blood platelets. In the case of the respiratory enzymes this problem is complicated further by the fact that the reaction occurs through the mediation of a series of enzymes, each of which is necessary to obtain oxygen uptake.

The Substrates of Endogenous Oxygen Uptake

Otto Heinrich Warburg[1] attributed most of the oxidations in the blood to the blood platelets because of their great number as compared to the relatively small number of leukocytes. However, this view is no longer entirely accepted. In previous studies[2] we have shown that the endogenous oxygen uptake of the individual horse blood leukocyte is at least 45 times that of a blood platelet. The Q_{O_2}† of the leukocyte was found to be -32.6, in contrast to -11.6 for rat liver slices. Also, there is another essential difference between leukocytes and liver, which have many enzymatic peculiarities in common; namely, the fact that the principal substrate for oxygen uptake in the liver is succinate, while for the leukocyte it is α-glycerophosphate.

The experiments on the identification of the substrates responsible for the endogenous uptake were performed on horse blood leukocytes; we have described the isolation of these cells and the enzymatic technique elsewhere.[2] A good test for the study of oxygen uptake is leukocyte dialysis against distilled water. After such dialysis the endogenous oxygen consumption in leukocyte suspensions is reduced almost to zero, but it can be restored by adding some substrates, such as succinate or α-glycerophosphate. There is good experimental evidence that both of these substrates are removed by dialysis. We examined the cell suspensions for the presence of the two substrates in undialyzed and dialyzed conditions, by paper chromatography according to the method of Hanes and Isherwood,[3] and by column chromatography for α-glycerophosphate as described by Khym and Cohn[4]; that is, by ion exchange with the use of the different borate fractions. For the fermentation acids, paper chromatography was done by the technique of van Duuren,[5] and column

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This is the sixth of a series of articles on enzyme studies on white blood cells and blood platelets.

† The symbol Q_{O_2} represents oxygen consumption in terms of the number of microliters consumed in 1 hour by 1 mg. (dry weight) of tissue. By convention the consumption of oxygen is given a negative value.

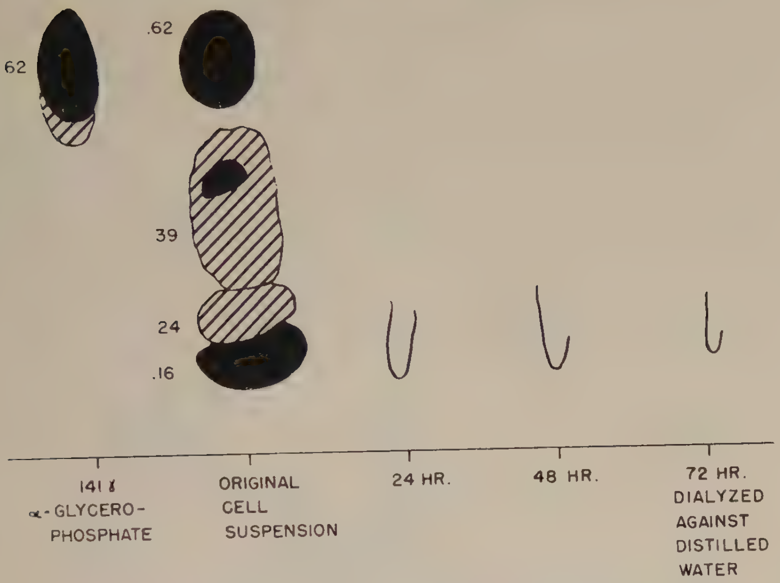
62
.62
39
24
.16
141 γ α-GLYCERO-PHOSPHATE
ORIGINAL CELL SUSPENSION
24 HR.
48 HR.
72 HR. DIALYZED AGAINST DISTILLED WATER

Figure 1. Leukocyte-platelet suspension. The material is dispersed, partially crushed, and deproteinized with perchloric acid. This chromatogram was performed by the method of Hanes and Isherwood.[3] The amount spotted is equivalent to 30×10^6 leukocytes.

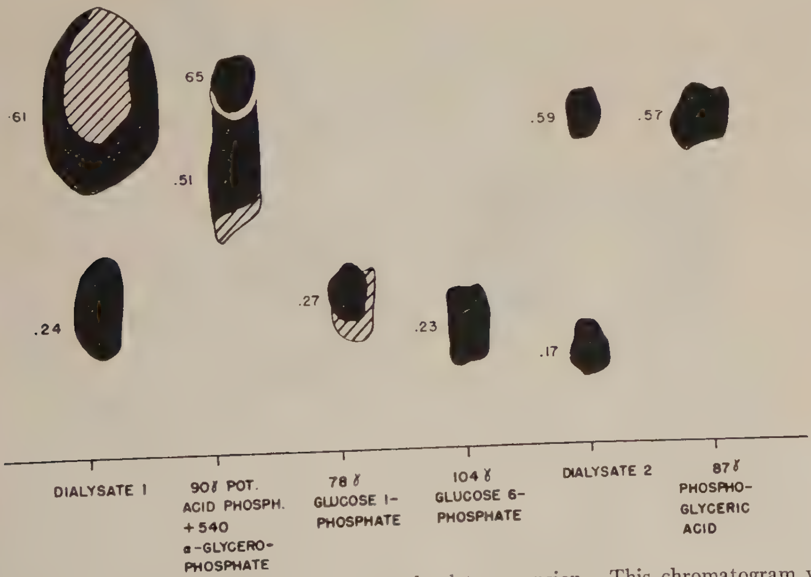
.61
65
.51
.59
.57
.24
.27
.23
.17
DIALYSATE 1
90γ POT. ACID PHOSPH. + 540 α-GLYCERO-PHOSPHATE
78 γ GLUCOSE 1-PHOSPHATE
104 γ GLUCOSE 6-PHOSPHATE
DIALYSATE 2
87γ PHOSPHO-GLYCERIC ACID

Figure 2. Dialysate of horse leukocyte-platelet suspension. This chromatogram was preformed by the method of Hanes and Isherwood.[3] The amount spotted is equivalent to 30×10^6 leukocytes.

chromatography by the method described by Neish.[6] The dialysates were also investigated for the two substrates.

The results are presented in the following illustrations. FIGURE 1 shows in the undialyzed suspension a spot of α-glycerophosphate and spots of some other phosphorylated intermediates. Their R_Fs are near those of glucose-1- and glucose-6-phosphate. In the dialyzed suspensions α-glycerophosphate is no longer detectable, and only faint spots of another intermediate can be seen, the R_F being near hexose diphosphate. On column chromatography α-glycerophosphate can be eluted together with glucose-6-phosphate.

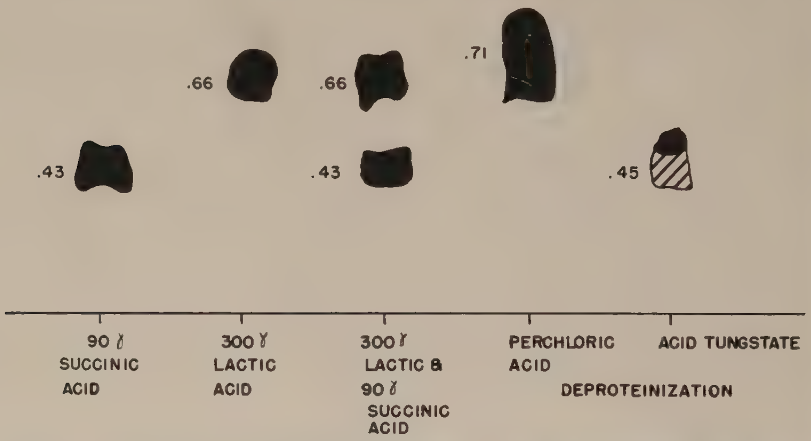

FIGURE 3. Horse leukocyte-platelet suspension. This chromatogram was performed according to the method of van Duuren.[5] The amount spotted is equivalent to 280 × 10^6 leukocytes.

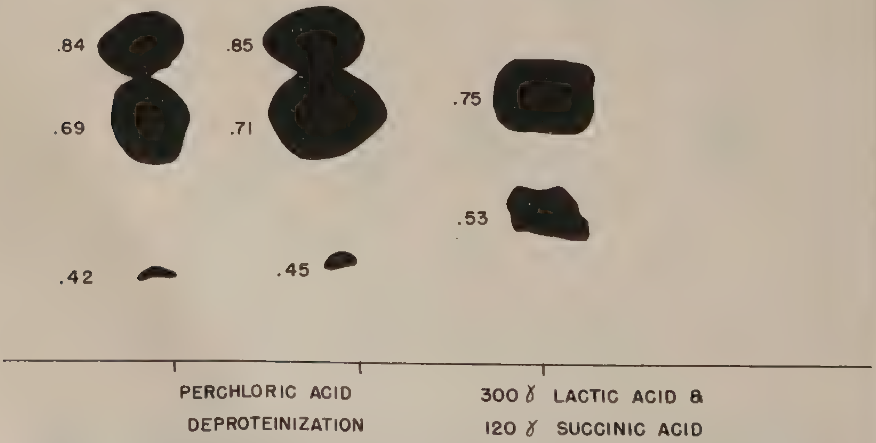

FIGURE 4. Horse leukocyte-platelet suspension. This chromatogram was performed according to the method of van Duuren.[5] The amount spotted is equivalent to 280 × 10^6 leukocytes.

FIGURE 2 is the chromatogram of two different dialysates. Glucose-6-phosphate and α-glycerophosphate were identified in the first, and phosphoglyceric acid was identified in the second. FIGURE 3 is a chromatogram made by the method of van Duuren.[5] Lactic acid is detectable with perchloric acid deproteinization, and succinic acid by acid-tungstate deproteinization. However, these reactions are not always reproducible; in most instances another acid was found, as shown in FIGURE 4. In addition to lactic acid, two spots appear that have an R_F lower than that of succinic acid. We have been unable to identify this acid to date. These spots occurred in many of our chromatograms and require further study.

FIGURE 5 is a chromatogram of a dialysate showing phosphoglyceric acid,

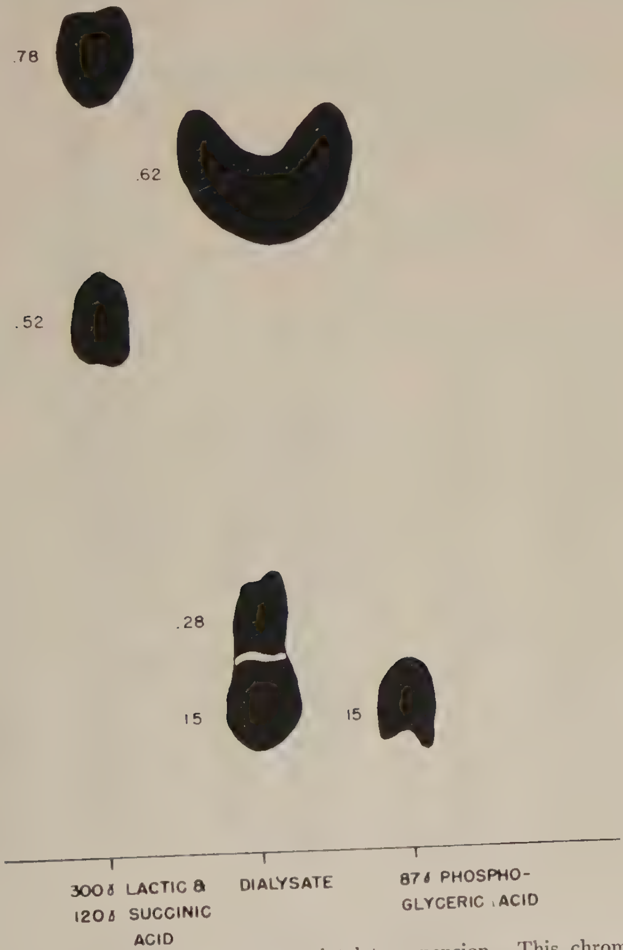

FIGURE 5. Dialysate of horse leukocyte-platelet suspension. This chromatogram was performed according to the method of van Duuren.[5] The amount spotted is equivalent to 30×10^6 leukocytes.

in analogy with the results shown in FIGURE 2, in which the acid was identified by the method of Hanes and Isherwood.[3]

These chromatographic studies may be considered proof that the loss of endogenous oxygen uptake following dialysis against distilled water is the result of a loss of substrates. The addition of the dialysate, sterilized with penicillin and streptomycin, to a dialyzed cell suspension did not restore the dehydrogenase activity. Obviously the concentration of the substrates is too low.

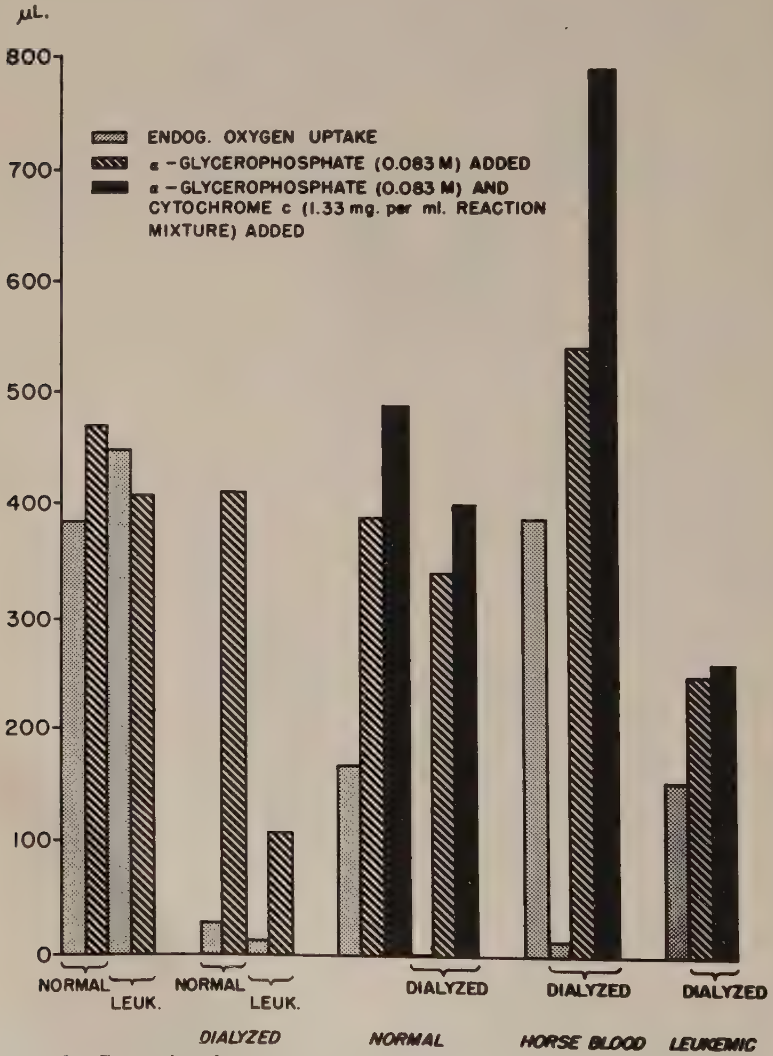

FIGURE 6. Comparison between normal and leukemic leukocytes: microliters of oxygen uptake per 1000 × 10^6 leukocytes in 90 min.

α-Glycerophosphate Dehydrogenase Activity in Leukemic Leukocytes

The technique of studying oxygen uptake on dialyzed cell material with added substrates has proved to be particularly informative in the study of leukocytes obtained from patients with chronic myelogenous leukemia. FIGURE 6 shows that the increase of oxygen uptake above the level of the endogenous respiration when α-glycerophosphate is used as substrate does *not* occur in leukemia when the cell suspension is undialyzed. When the cell material is dialyzed the oxygen uptake of leukemic cells is only approximately 25 per cent that of normal human white blood cells. In the third group of bars one can see that addition of cytochrome *c* in excess considerably increases α-glycerophosphate dehydrogenase activity in both undialyzed and dialyzed normal human leukocytes. In undialyzed horse blood leukocytes this increase is missing.[2] However, in horse blood leukocytes there is a considerable increase in activity if the cells are dialyzed. *In dialyzed leukemic leukocytes, there is no increase of α-glycerophosphate dehydrogenase activity following addition of cytochrome c to the reaction mixture.* Addition of cytochrome *c* without α-glycerophosphate to dialyzed human or horse leukocytes does not increase oxygen uptake.

We cannot conclude from our studies that it is the α-glycerophosphate dehydrogenase activity which is at a lower level in leukemic leukocytes. The defect can be in any of the series of enzymes necessary to obtain oxygen uptake. The behavior of leukemic leukocytes may be due to an actual difference in the cytochrome concentration in the cells or it may be the result of differences in the extractibility of cytochrome *c*. Quantitative cytochrome determinations are in progress.

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MYELOPEROXIDASE*

By Julius Schultz
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The principal difficulty that impeded previous investigations of myeloperoxidase (also called verdoperoxidase) has been the limited sources of this enzyme. Agner[1] discovered peroxidase in the leukocytes of tubercular empyema, later showed that it was present in the blood of the chloroleukemic patient, and observed it spectroscopically in a 27-year-old specimen of chloroma. Other investigators have continued these studies from other sources. Foulkes, *et al.*[2] reported myeloperoxidase from nontubercular empyema, and Chance[3] demonstrated the enzyme in leukocytes obtained by the interperitoneal injection of saline into sensitized rabbits. Maehly[4] recently reported the difficulties in obtaining the enzyme from ox blood and human leukocytes. The limited availability of the material, as well as the low recovery of the enzyme, seriously handicapped progress in this field.

The development of the experimental leukemic tumor, chloroma, in the rat made available a rich source of myeloperoxidase from which the present studies stem. These included the demonstration of the presence of this enzyme by isolation and characterization, its crystallization for the first time, its localization in the chloroma cell, its relation to other hemoprotein enzymes, and factors influencing its activity.

The chloroma is a green tumor known for more than a century to be present in certain cases of myelogenous leukemia in man. Experimental chloroma in the rat was shown to be akin to the human tumor[5] in that it fluoresces red under ultraviolet light and is green in its natural state. The red fluorescence has been shown to be due to protoporphyrin, coproporphyrin, and at least 11 other chromatographic entities.[6] The green color is due principally to myeloperoxidase.

Concentration of myeloperoxidase in chloroma. While leukocytes were shown by Agner[1] and by Chance[3] to contain as much as 2 per cent of the dry weight of the cell, chloroma contains up to 6 per cent of the dry weight in tumors of young rats and as little as 0.4 per cent in older rats, as shown in TABLE 1.

While the concentration of the enzyme would indicate that large amounts were present, isolation methods indicate that either great losses took place, or that activators were present that would account for higher activities than indicated by the amount of enzyme available for isolation.

Distribution of myeloperoxidase and succinoxidase in cellular particulates of chloroma. In carrying out experiments on tissue fractionation of chloroma by the Schneider-Hogeboom[7] procedure, it was found that the peroxidase activity was not distributed in the same manner as succinoxidase, as seen in TABLE 2, and that activators were present in the mitochondrial supernatants. Two-hour centrifugation at 20,000 g resulted in a supernatant fraction that, when

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TABLE 1

PEROXIDASE ACTIVITY AND CONTENT OF CHLOROMAS OF VARIOUS AGES*

Chloroma				Change in body weight of tumor-free rat
Age	Weight	Peroxidase		
		Activity	Percentage of tumor weight	
(days)	(grams)	(K/mg.)	(grams)	(grams)
60	9.1	3.7	1.22	70
76	88.0	1.9	0.62	96
77	64.0	3.0	0.99	56
82	69.5	1.25	0.41	109
83	31.0	1.22	0.40	112
89	40.1	0.82	0.27	106
196	45.0	1.15	0.37	200
270	54.0	0.75	0.24	194
321	56.0	0.26	0.08	230
329	67.0	0.41	0.13	160

* The chloromas of the rats in this table were produced by the injection of the blood of a chloroleukemic rat. Age refers to the time of injection to the day of sacrifice. Peroxidase activity, K, was measured on fresh homogenates as described in the text. To obtain the figures in the fourth column the weight of the enzyme was calculated from activity where 300 K = 1 mg. enzyme.

TABLE 2

DISTRIBUTION OF MYELOPEROXIDASE AND SUCCINOXIDASE IN PARTICULATE MATTER OF CHLOROMA*

Fraction†	Total enzyme		Specific activity		Ratio	Total protein
	MPO	SD	MPO	SD	MPO/SD	(mg.)
Hom.	6660	2880	8.6	3.70	2.26	774
Nw_1	905	522	14.8	6.35	2.32	82
Mw_2	825	575	14.5	9.95	1.44	58
S_1	3550	620	5.8	1.10	5.80	580
P_1	2420	279	16.8	2.18	7.30	130
S_2	1240	0	2.7			455

* Particulate matter prepared and named according to Schneider procedure, using sucrose. MPO refers to myeloperoxidase activity calculated from velocity, $K = \sqrt{1/t}/ml.$ (t = time in seconds). SD is succinoxidase activity as measured by change in O.D. per minute at 550 following addition of succinate. Specific activity refers to activity/mg. protein. Total enzyme is calculated from the activity per ml. of fraction and the total volume of that fraction.

† Symbols: Nw_1, nuclear fraction; Mw_2, mitochondria fraction; S_1, mitochondria supernatants; P_1, pellet obtained after continued centrifugation of S_1; and S_2, supernatant of P_1.[7]

added to chemically purified enzyme, resulted in at least 50 per cent increase in activity, as shown in FIGURE 1. TABLE 3 shows that the microsomal fraction contains material with an absorption peak at 430 mμ that is associated with the activator, and that even in chemically purified fractions of peroxidase the presence of higher activity is associated with this particular peak (see Chart 4 of Schultz *et al.*[8]).

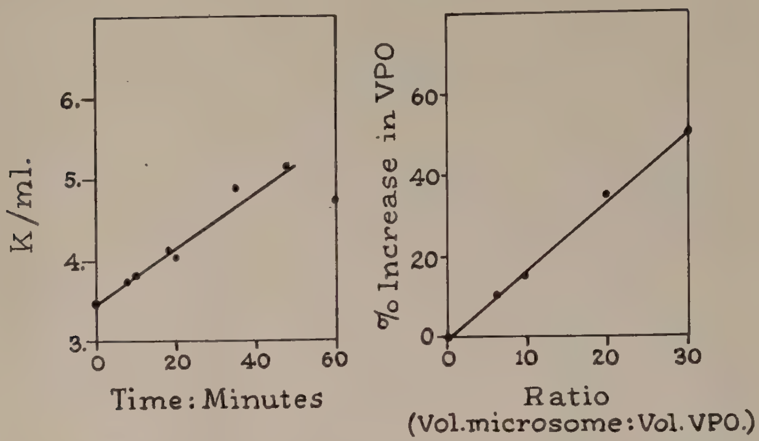

FIGURE 1. Left: Equal volumes of chemically purified VPO and microsomal fraction were mixed at room temperature, and the VPO activity (K) was measured at the time intervals indicated. Right: Various proportions of VPO and the microsomal fraction as indicated in the chart were mixed and allowed to stand 30 minutes before peroxidase activity was measured. Reproduced by permission of *Cancer Research*.[8]

TABLE 3
RELATION OF CHANGE IN O. D. ON REDUCTION AT 470 Mμ AND 430 Mμ TO VPO ACTIVITY

Source	K^0	470×10^3	$K^0/470 \times 10$	430×10^3
20E*	100.0	200	5.0	0
T-F_2*	10.8	31	3.6	0
T-4*	62.8	114	5.5	0
9a*	18.3	45	4.0	5
Micros. A	4.5	5	9.0	70
Micros. B	31.0	30	10.0	135
Mitochondria	61.2	165	3.7	0
		$K^0 = \sqrt{1/t/ml.}$		

* Sources of material tested refer to purified preparations obtained by chemical fractionation.

Isolation and crystallization of myeloperoxidase. The myeloperoxidase of chloroma can be visualized readily by a reduced-difference spectrum, as seen in FIGURE 2. This is similar to the reduced-difference spectrum obtained by Chance[3] in the enzyme from leukocytes obtained by the interperitoneal injection of saline into sensitized rabbits. Note that the isosbestic points* are at 450, 500, and 580, which are identical with those of Chance[3] and of Agner.[1] We have found an identical curve in a fresh post-mortem sample of chloroma from a human case of myelogenous leukemia, where the spectrum was obtained on an homogenate within an hour after autopsy.

When chloroma tissue is fractionated according to the procedure for isolation of myeloperoxidase as originally described by Agner,[1] the products ob-

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* Isosbestic points are those wave lengths at which the optical densities of the oxidized and reduced spectra of a given substance are equal.

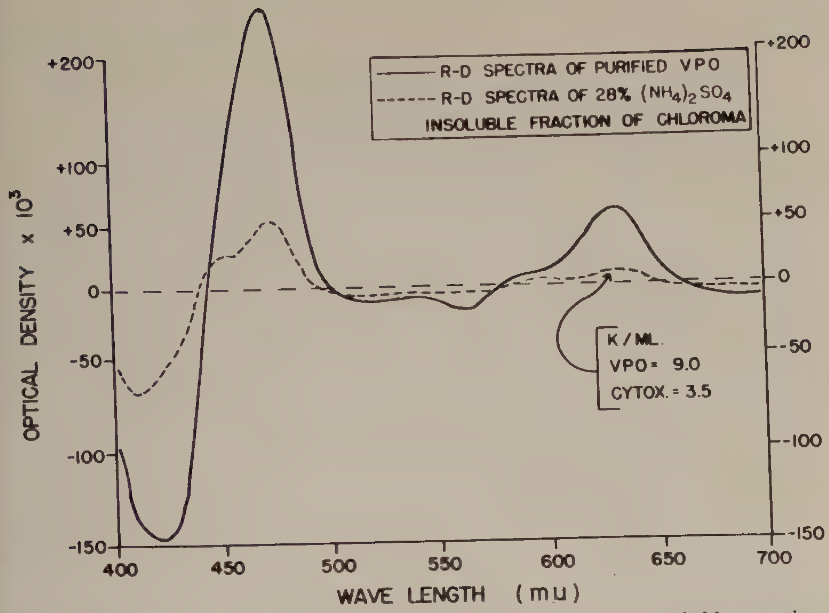

FIGURE 2. Reduced difference spectrum of purified myeloperoxidase of chloroma tissue is shown by the solid line. The dotted line is that of a fraction containing some peroxidase, but also containing cytochrome oxidase ($a + a_3$). The isosbestic point at 450, 500, and 580 mμ of the myeloperoxidase is identical with that of Chance[3] and Agner[1].

tained were practically identical to Agner's, as seen in FIGURE 3; this procedure does not lead to products capable of crystallization. By devising a column chromatographic procedure,[9] and avoiding the use of alcohol, dialysis against water, and electrophoresis, it was possible to obtain preparations whose spectrum can be seen in FIGURE 4. When these fractions were repeatedly precipitated with ammonium sulfate, redissolved, dialyzed against increasing concentrations of the same salt, and subjected to slow evaporation, the crystals seen in FIGURE 5 were obtained.

It is of interest to note that prior to the work on experimental chloroma there was controversy among some investigators in France[10, 11] as to the origin of the red fluorescence, the green color, and the relation between them. One opinion[10] was based on the fact that some porphyrin-protein complexes could fluoresce red and still be green in color, and that peroxidase had nothing to do with the green color, while the opposing view[11] was that the green color could be the enzyme and that the fluorescence was due to porphyrin. However, in the absence of isolation of pure products there could only be speculation. Now we have isolated crystalline protoporphyrin,[6] the chief agent responsible for the red fluorescence, and we have crystallized the green enzyme myeloperoxidase which, because of its color, is also called verdoperoxidase,[12] but these latter crystals are red fluorescent, and to date efforts to separate the red fluorescent porphyrin from the green crystalline enzyme by physical means have been unsuccessful.

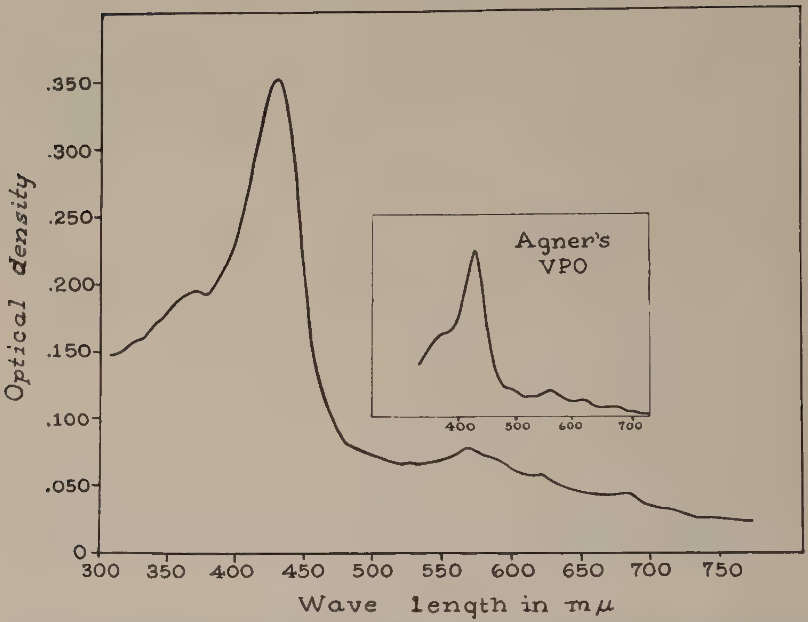

FIGURE 3. Absorption spectrum of a green pigment obtained from rat chloroma tissue using Agner fractionation procedure for VPO. Reproduced by permission of *Cancer Research*.[5]

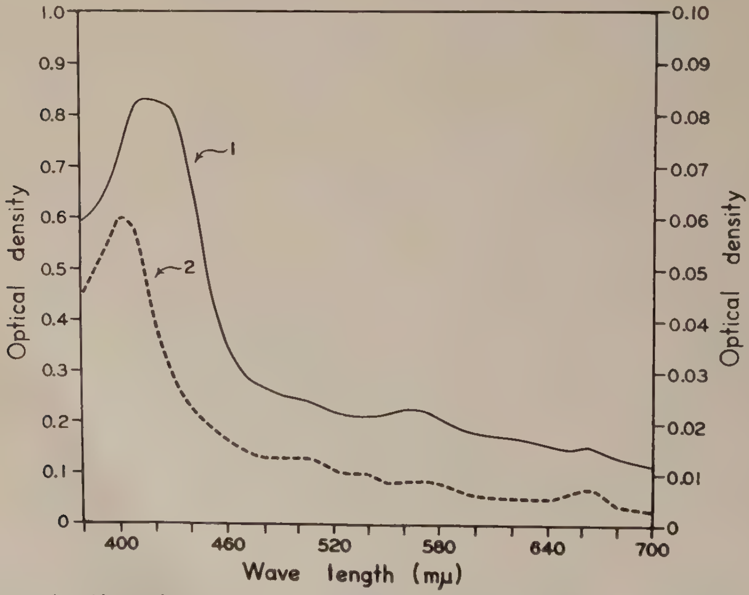

FIGURE 4. Absorption spectrum of a myeloperoxidase preparation. Curve 2 is the prophyrin removed by acetic acid and ether. Right-hand scale 0.000–0.100 O.D. refers to curve 2, and left-hand scale to curve 1. Reproduced by permission of the *Journal of the American Chemical Society*.[9]

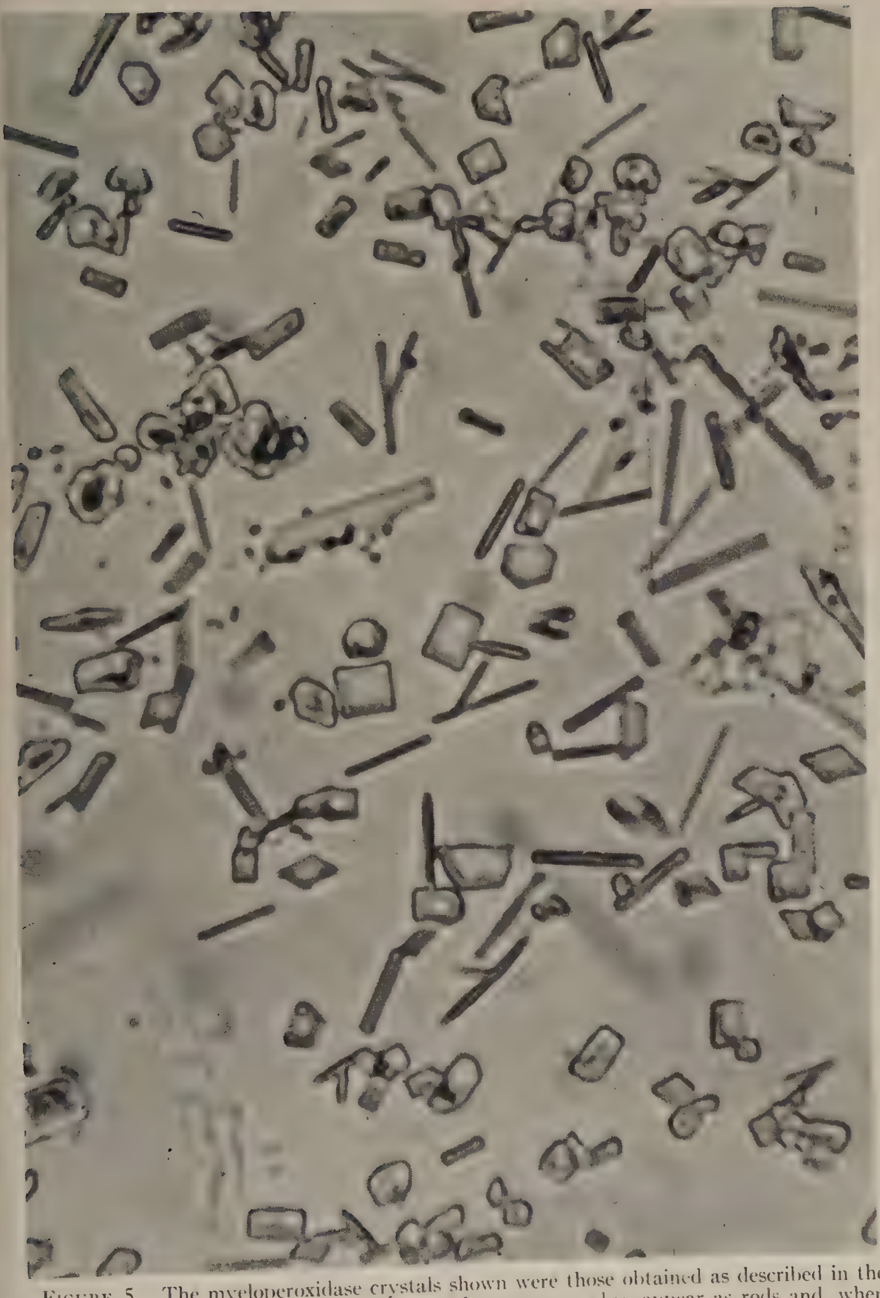

FIGURE 5. The myeloperoxidase crystals shown were those obtained as described in the text. They are thick hexagonal plates that when seen on edge, appear as rods and, when viewed from the side, are seen as rectangles, thus accounting for all of the forms visible in the field. They are strongly red fluorescent and have an absorption spectrum similar to that seen in FIGURE 4.

TABLE 4

MYELOPEROXIDASE, HEME-ENZYME SYSTEMS, AND HEMOPROTEIN DERIVATIVES OF CHLOROMA

Substance	Units used†	Range of values
MPO	mg./gm.	1.0–10.0
Succinoxidase	μM cytochrome *c** × min. − 1 × gm. − 1	0.15–0.5
DPNH Cyt. *c* reductase	μM cytochrome *c* × min. − 1 × gm. − 1	4.5–
Cytochrome oxidase	μM cytochrome *c* × min. − 1 × gm. − 1	5.0–7.5‡
Cytochrome *c*	μg./gm.	5.0–19.0
DPN	μg./gm.	161.0–260.0
DPNH	μg./gm.	26.0–72.0
Hematin	μg./gm.	26.0–
Protoporphyrin	μg./gm.	10.0–18.0
Coproporphyrin	μg./gm.	0.4–0.9
x-Porphyrins (5)	μg./gm.	0.1–1.2

* Rate of cytochrome *c* reduced.
† Rate of cytochrome *c* oxidized.
‡ In the presence of CO activity is reduced to 0.

The porphyrin responsible for the fluorescence of the crystals can be removed chemically, but the maximum peroxidase activity appears to be associated with the presence of this porphyrin. Consequently, the problem of the green color and red fluorescence is still with us.

Relationship of verdoperoxidase to other hemoprotein enzymes of chloroma tissue. The chloroma tissue cell contains the principal members of the cytochrome system (TABLE 4). In general, the values of cytochrome oxidase are about two thirds that of normal liver, and greater than that generally found in tumors. In the presence of carbon monoxide this is reduced to zero, which indicates that peroxidation of cytochrome is not taking place. Cytochrome *c*, diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), and succinoxidase are about the same as that found in tumors generally. FIGURE 6 shows a relation of myeloperoxidase to succinoxidase in a series of growing tumors. This indicates some degree of order in the growth process taking place and implies that the peroxidase is not overproduced in respect to other enzyme systems, for with each micromole of peroxidase present per 100 gm. of tissue, there is a fifteenfold increase in the ability of succinate to reduce the concentration of oxidized cytochrome *c*, at a rate of 1 μM per minute, thus reflecting a fifteenfold increase in activity of that portion of the electron transport system per 100 gm. of tissue. Whether this reflects a purely morphological phenomenon or indeed suggests a possible connection of myeloperoxidase in electron transport system may be more clearly understood in future studies. This calls to mind the series of reactions proposed by Lenhoff and Kaplan[13] wherein the peroxidase of *Pseudomonas fluorescens* oxidizes reduced cytochrome *c* (formed from the reaction with DPNH) in the presence of hydrogen peroxide (formed from the reaction of DPNH and flavo-protein). Myeloperoxidase can oxidize DPNH very rapidly in the presence of H_2O_2 (unpublished data), but the relation of this to the flavo-protein reaction is yet to be examined.

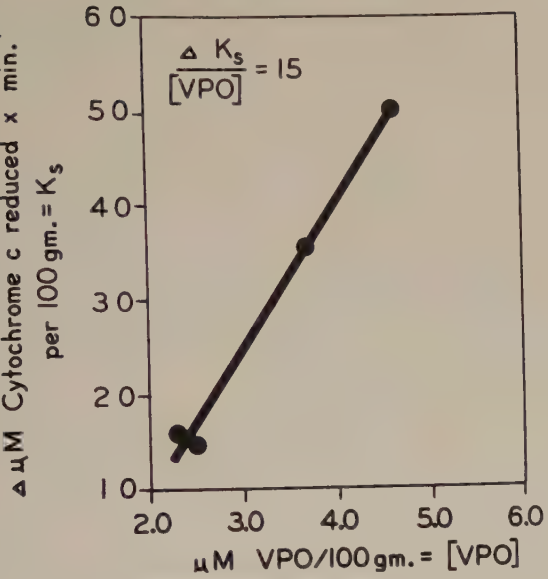

FIGURE 6. Variation of succinoxidase activity with VPO activity of 4 chloromata.

Summary. It is assumed here that all of the sources, mentioned earlier, from which the green peroxidative enzyme has been found present by isolation and spectrophotometric characterization are rich in the granulocytic cells of the myeloid series, although these were not the only cells present. In chloroma, a leukemic tumor of the myeloid series that is analogous to the lympoid tumor of lymphatic leukemia, nature has provided a cell truly rich in material for study. This tumor, when produced experimentally, has made possible the isolation and crystallization of the characteristic enzyme of this type of cell. Although crystals contain a fluorescent contaminant, this is not new in the case of hemoprotein enzymes. In fact, myeloperoxidase owes its illumination to the fact that the crystalline preparation of catalase first reported by Sumner and Dounce[14] was contaminated with a green fluorescent material. Agner,[1] believing this to have arisen from hepatic bile pigments, tried to isolate catalase from a nonhepatic source, such as the leukocyte, and instead discovered verdoperoxidase, which he later named myeloperoxidase. Lenhoff and Kaplan[13] reported red fluorescent cytochrome *c* preparations from *Pseudomonas fluorescens* that they have yet to identify.

Although the metabolic role of peroxidative enzymes is not exactly clear in nature itself, much less within the leukocyte, Agner[15] has assigned to the green peroxidase of the myelocyte the teleological role of clearing the animal body of toxins, while Canellakis *et al.*[16] have reported the dramatic degradation of uric acid in the presence of this enzyme, and Chance[3] claims that cytochrome a_3, rather than this enzyme, deserves the distinction of participating in the electron transport system of the leukocyte. This leaves the previously mentioned findings of Lenhoff and Kaplan[13] as the one possible approach to gaining an insight to the role of this peroxidative catalyst in the metabolic scheme of this mammalian cell, the function of which we are only now beginning to understand.[17]

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LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY IN THE RAT*

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Alkaline phosphomonoesterase is a highly ubiquitous and interesting enzyme that is widely distributed in animal and human tissues. In the normal rat strongly positive alkaline phosphatase activity can be demonstrated in 70 to 90 per cent of the segmented neutrophils in the peripheral blood. This marked leukocyte alkaline phosphatase (LAP) activity is also found in the hamster, the burro, the steer, the guinea pig, and the rabbit. Normal human leukocytes contain relatively little of this enzyme and even greater deficiency is found in the mouse, the cat, and the dog. In all species examined, monocytes, lymphocytes, and erythrocytes were devoid of activity; however, eosinophils in the rat, the burro, and the steer contain this enzyme.

An intriguing feature is the variation of LAP activity in health and disease.[1, 2] In humans, following pyogenic infections or in disorders such as myeloid metaplasia, LAP is greatly increased. In contrast, in patients with chronic myelogenous leukemia neutrophils contain almost no activity. Interestingly enough, J. W. Hollingsworth and S. C. Finch (personal communication) recently found that in chloroleukemia of the rat (Shay) the leukemic myeloid cells were strongly LAP positive. We have confirmed this finding by histochemical, biochemical, and electrophoretic studies.

Bone marrow smears, crowded as they are with immature myeloid and erythroid cells, are usually unsatisfactory for histochemical procedures. However, in normal rat marrow, myeloblasts, promyelocytes, myelocytes, and metamyelocytes show strong LAP activity. In human marrow only a small percentage of granulocytes except for an occasional histiocyte or osteoblast, are positive.

Rat LAP is similar in many characteristics to other alkaline phosphomonesterases; however, certain important differences have been noted (G. Brecher, personal communication). Human LAP is inhibited by the chelating effect of ethylenediaminetetraacetic acid sodium (EDTA);[3] this does not occur with the rat or the rabbit.[4] In this connection a practical point should be noted. When separated intact human leukocytes are collected in EDTA-containing solutions, no inhibition occurs if the cells are washed before homogenation and incubation with substrate. On the other hand, if EDTA is added to homogenated human leukocytes there is a marked inhibition of LAP activity; this does not take place with homogenated rat leukocytes. This difference in EDTA effect also can be demonstrated histochemically.

While a good deal is known concerning the enzymatic characteristics and behavior of LAP, the mechanisms controlling the concentration and activity of this enzyme *in vivo* are not well established. Valentine and his co-workers[5] have shown that LAP activity markedly increases in humans during situations

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of stress, as during pyogenic infections following burns, in myocardial infarction, and postoperatively. Following the administration of ACTH and, to a lesser degree, with large doses of cortisone, LAP activity is increased greatly in normal individuals.

In Addison's disease the administration of ACTH was not followed by a rise in LAP activity, but an increase did occur with large doses of hydrocortisone.[6] However, Valentine suggests that, while the adrenal mechanism may be one pathway for the control of LAP activity, it is probably not the only one. He has also pointed out that this postulated endocrine relationship does not extend to all species, since the dog and cat show no LAP response to ACTH.[7]

Certain aspects of alkaline phosphatase activity have been studied extensively *in vitro*. Investigations of this sort furnish technical information and also provide insight into the chemical nature and kinetics of this enzyme. However, many experiments, including some of our own,[8] have been carried out under unphysiological conditions, and such data may be misleading or unrelated to what actually takes place in the organism. The fundamental problems concerning the biochemical and physiological role of LAP require investigation *in vivo*. Using the rat as an experimental animal, observations of the effect of various agents on LAP activity were carried out. It was found that total-body X ray at various dose levels increased LAP activity. To determine the role of the adrenal, rats were adrenalectomized and, following this procedure, exposed to total-body irradiation. The results of these preliminary experiments are presented below.

Methods and Materials

Initial studies were made on rats to determine the range of leukocytes and differentials in peripheral blood. Whole blood specimens (0.5 ml.) were collected in heparin-rinsed glassware from the tail of the animal and mixed with EDTA and dextran solution. Cells were separated, washed, and resuspended as described in a previous publication.[4] Biochemically, LAP was determined by the method of Valentine and Beck[2] and, in many cases, duplicate studies were carried out using the Bessey-Lowry[9] technique. Values were expressed as milligrams of P or of phenol released in 1 hour by 10^{10} leukocytes or 10^{10} polymorphonuclear leukocytes (polys.). LAP was confirmed also by histochemical methods on blood smears.[10, 11]

Rats were exposed to varying doses of total body irradiation, beginning with 50 r (250 kvp, 15 mAmp, Thorasi filter, HVL 2.3 mm. cu. distance 70 cm. 1¼ minutes for 50 r). A total-body dose of 400 r was selected as having an optimal effect on the leukocytes while allowing for the recovery of the rat.

A group of 16 male Wistar rats each weighing from 190 to 200 gm. was irradiated in this fashion. Leukocyte counts, differentials, and determinations of LAP activity were carried out prior to and on the fifth, tenth, sixteenth, and twenty-second days after irradiation.

For the experiments on the effect of adrenalectomy, after preliminary trials, a series of male Wistar rats were adrenalectomized and maintained on salt and water. Studies on leukocytes and LAP activity were carried out prior to adrenalectomy and at weekly intervals thereafter. After 4 weeks, 10 surviving rats were exposed to 250 r and 6 others to 400 r. An additional series of 23 rats was given 400 r 1 week following adrenalectomy. Studies on leukocytes and LAP activity were carried out on the fourth, eleventh, and twenty-first days following irradiation.

Experimental Results

The effect of 400 r total-body irradiation on LAP activity was as follows. The expected fall in leukocytes occurred in the animals. As shown in FIGURE 1, the LAP activity was elevated on the fifth and fell to within normal limits by the tenth postirradiation day. In preliminary experiments, a rise in LAP activity was found to occur usually after the second postirradiation day and reached a peak between the fourth and sixth days. Since daily withdrawals of blood resulted in anemia and infection of the tail, longer intervals were selected for bleeding.

In any event, in this and other experiments 400 to 500 r and, to a less marked degree, 250 r total-body irradiation produced a significant rise in LAP activity in the polys. by the fifth day postirradiation.

Effect of adrenalectomy on LAP activity. In the first series of 16 rats, follow-

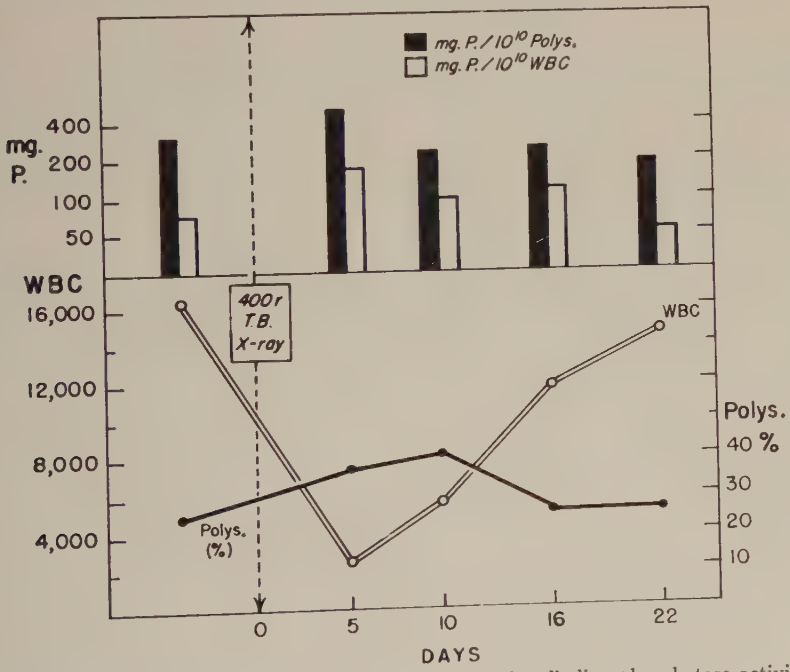

FIGURE 1. The effect of 400 r total-body X ray on the alkaline phosphatase activity of leukocytes and polymorphonuclear leukocytes of the peripheral blood in the rat (18 male Wistar rats weighing from 190 to 200 gm. each).

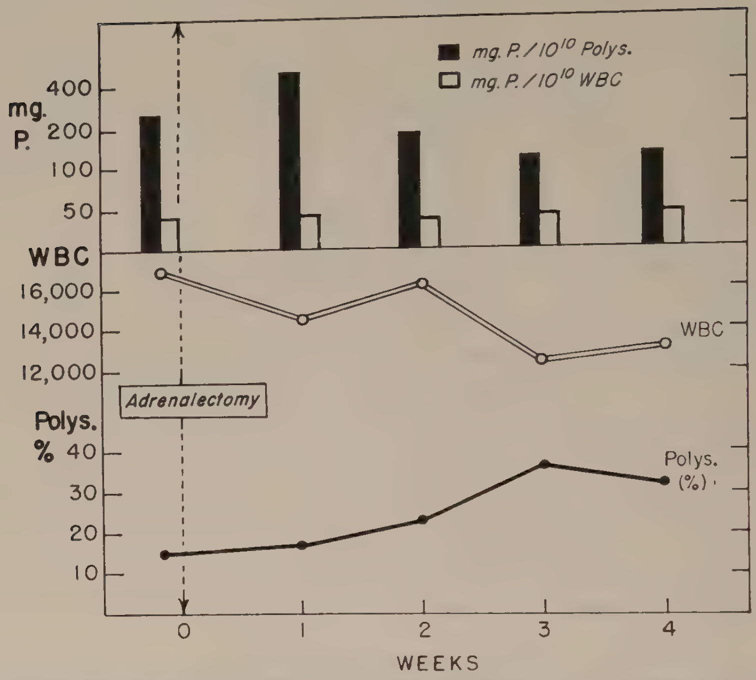

FIGURE 2. Effect of adrenalectomy on the alkaline phosphatase activity of the leukocytes and polymorphonuclear leukocytes in the rat (16 male Wistar rats weighing from 190 to 200 gm. each).

ing removal of the adrenals, a marked rise in LAP activity was observed in the polys. by the seventh postoperative day. Subsequently this activity returned to a normal level (FIGURE 2).

In the second series of 23 adrenalectomized rats, studies on the seventh postoperative day also showed a high level of LAP activity in the polys.

Effect of 400 r total-body irradiation on the LAP of adrenalectomized rats. In 9 rats surviving 4 weeks after adrenalectomy the LAP activity rose from 174 mg. to 215 mg. per 10^{10} polys. 5 days following 250 r. By the eleventh day LAP activity had dropped to 143 mg. per 10^{10} polys. After a 400 r dose to 6 rats surviving 4 weeks postadrenalectomy, poly.-LAP activity was markedly elevated by the fifth postirradiation day.

In another group of 23 rats, following 400 r irradiation 1 week after adrenalectomy, a marked rise in LAP activity in the polys. was observed on the fourth postirradiation day. As shown in FIGURE 3, this elevation disappeared by the eleventh day and, in the surviving rats, LAP activity returned to normal limits. Mortality was high in this group, and 16 rats survived to the fourth day, but only 4 rats were alive on the twenty-first postirradiation day.

Discussion

The effect of total-body irradiation has been studied extensively in the rat. Together with other fundamental observations, the work of Patt and his

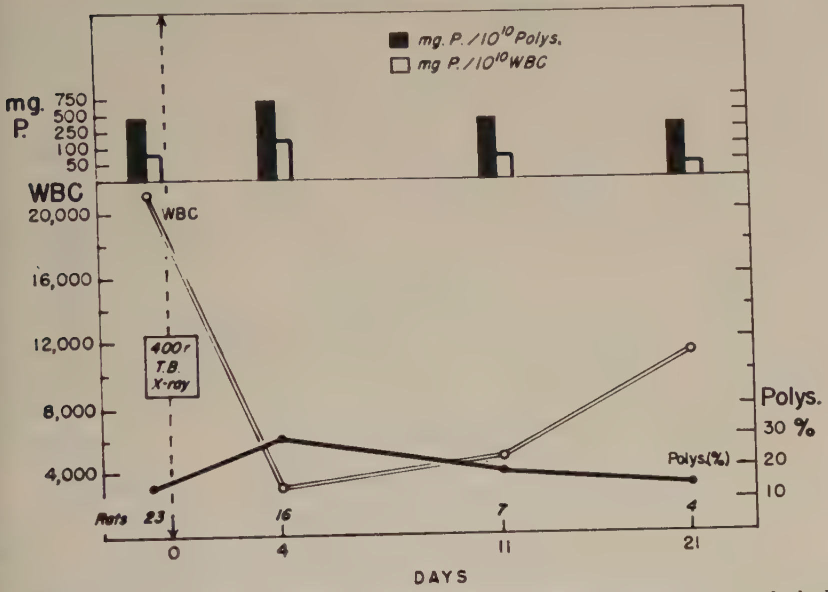

Figure 3. Effect of 400 r total-body X ray, 7 days following adrenalectomy, on the leukocyte and polymorphonuclear leukocyte alkaline phosphatase activity of the rat.

associates[12] indicated that a considerable adrenal response was elicited by total-body irradiation, and this concept has received support from other investigators. In the present study the expected fall in leukocytes occurred and was accompanied by a significant rise in LAP activity. This might seem to be due to adrenal cortical stimulation. However, Betz[13] has noted that in rats the regeneration and repair of destroyed hematopoetic tissues is evident on the fourth postirradiation day. It is possible that products of tissue breakdown and processes of repairs may stimulate LAP activity via some pathway other than the adrenal cortical axis.

In these experiments removal of the adrenal did not cause a decrease in LAP activity. Instead, enzyme activity rose in the polys. and subsequently fell to within normal limits. Furthermore, rats responded to total-body irradiation with an increase in poly.-LAP activity following adrenalectomy.

These experiments do not unequivocally rule out the role of the adrenal in the control of LAP activity in the rat. In 4 surviving rats residual adrenal tissue was found at autopsy by microscopic examination. Although histological studies were made on extirpated adrenals, it is evident that small remnants of capsule and gland may undergo regeneration. However, death within a few days followed the withdrawal of saline, and this seems good evidence of successful adrenalectomy in most of these animals.

Summary and Conclusion

The polymorphonuclear leukocytes (polys.) of rat peripheral blood contain strong leukocyte alkaline phosphatase (LAP) activity, and this can be demonstrated readily by histochemical and biochemical methods.

Following total-body irradiation, LAP activity increased markedly and later returned to normal levels.

Adrenalectomy in the rat did not cause a fall in LAP activity; instead the enzyme activity in the polys. rose and later returned to normal.

When adrenalectomized rats received 400 r total-body irradiation, the poly.-LAP activity increased markedly.

These experiments suggest that, in the rat, control of LAP activity is not related to the adrenal, but must be mediated through some other mechanism.

Acknowledgments

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DIFFERENCES IN NUCLEIC ACID METABOLISM BETWEEN NORMAL AND LEUKEMIC HUMAN LEUKOCYTES*

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There can be little doubt that processes of cell division and of growth are intimately interwoven with biochemical reactions that involve the synthesis and function of nucleic acids, and there is considerable reason to suspect that some of the agents that prolong the lives of patients with leukemia do so by virtue of some effect on the formation or function of the nucleic acids. Considerations such as these have led us to initiate studies on the nucleic acid metabolism of leukocytes and to search for exploitable differences between the metabolism of normal cells and those from patients with various types of leukemia.

In the course of this work we have found that leukocytes are a most satisfactory material for fundamental biochemical studies. They can be obtained in reasonable quantities almost undamaged, they have an active metabolism, and they can be studied in their native environment, in which they continue to be active for considerable periods of time. There are, of course, complexities in the use of these cells for metabolic studies; one such problem is the heterogeneity of cell types found in most preparations.

In presenting this paper I speak for a group of chemists and clinicians[2] at the University of Illinois that is investigating the metabolism of normal and leukemic leukocytes. Our approach to the problem of their nucleic acid metabolism has been to study the incorporation of radioactive precursor compounds into isolated leukocytes incubated in serum containing the labeled compound, and to investigate the effects of several inhibitors on the incorporation of these precursors. The radioactive precursors that we have used most extensively are shown in TABLE 1.

We have studied the effects of a number of clinically effective agents on the incorporation of these precursors into the protein and nucleic acids of leukocytes. These agents include the folic acid antagonists, the purine antagonists, the pyrimidine antagonists, the amino acid antagonists, and the bifunctional alkylating agents. TABLE 2 lists the most extensively studied agents.

During the course of this work we have observed a good many striking differences between the metabolism of leukocytes of different types, and it is with some of these differences that this paper is concerned. It has become evident that leukocytes of different types are at least as different metabolically as they are morphologically.

We have described our methods for isolation and incubation of the leukocytes from blood previously.[1, 2] Isolation involves the use of fibrinogen to hasten sedimentation of the erythrocytes, as described by Beck and Valentine.[3] The leukocytes were collected by low-speed centrifugation, washed with saline,

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* The work reported in this paper was supported by grants C-1828 and C-2347 from the National Cancer Institute, Public Health Service, Bethesda, Md.

TABLE 1

RADIOACTIVE NUCLEIC ACID PRECURSORS

Formate-C^{14}
Glycine-1-C^{14}
Glycine-2-C^{14}
Serine-3-C^{14}
Adenine-8-C^{14}
Guanine-8-C^{14}
Hypoxanthine-8-C^{14}
Orotic acid-2-C^{14}
Uracil-2-C^{14}
Thymine-2-C^{14}

TABLE 2

CHEMOTHERAPEUTIC AGENTS STUDIED *IN VITRO*

A-Methopterin
Daraprim
Pteroyl aspartic acid

6-Mercaptopurine
6-Chloropurine
Thioguanine
2,6-Diaminopurine
Purine riboside

5-Fluorouracil
5-Fluoroorotic

Azaserine
DON
Selenium cystine

Myleran
Chlorambucil

and resuspended at a concentration of 1 × 10^8 cells (about 2.5 mg. dry weight) per milliliter of fresh normal serum. They were then incubated at 37° C. under an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide in the presence of the isotope or inhibitor under study, usually for a period of 4 hours. The cells were then collected by centrifugation, washed with saline, and the proteins were precipitated with trichloracetic acid and extracted with lipid solvents to give the gross protein fraction (GPF). In those investigations in which the distribution of the label in the various components of the cell was studied in detail, the GPF was fractionated by what are now conventional methods involving column chromatography, ionophoresis, and paper chromatography to obtain products that had constant specific activity through at least two purification steps. We found that the incorporation of all of the precursors studied was linear with time for periods of at least 4 to 6 hours, and that uptake was maximal at the concentrations of precursors used.

Early in this work we observed that leukocytes have rather reproducible and characteristically different rates of incorporation of isotopic protein and nucleic acid precursors into the gross protein fraction (TABLE 3).

These data show that the rates of formate incorporation into normal, chronic lymphatic leukemia (CLL), chronic granulocytic leukemia (CGL), and acute leukemia cells rise, in that order. The sequence is similar for the other isotopic precursors. The incorporation of uracil was the least, and of glycine the greatest, in all types of cells. These differences in uptake rates represent the net result of differences in the components into which the precursors are incorporated, in precursor pool sizes and in metabolic pathways.

A second distinction among leukocytes of different types is their sensitivity

TABLE 3

INCORPORATION OF RADIOACTIVE PRECURSORS OF PROTEIN AND NUCLEIC ACID INTO DIFFERENT TYPES OF LEUKOCYTES*

	Normal	Chronic lymphaytic	Chronic granuloctic	Acute
		(CLL)	(CGL)	
Uracil-2-C^{14}	2.41 ± 0.6	13.3 ± 2.5	14.8 ± 3.0	—
Formate-C^{14}	5.5 ± .55	14.9 ± 1.0	27.1 ± 2.4	80.6 ± 8.1
Adenine-8-C^{14}	7.7 ± 1.4	47.9 ± 9.8	69.7 ± 20	—
Glycine-1-C^{14}	107 ± 16	301 ± 35	500 ± 61	—

* Results expressed as micromoles of precursor incorporated × 10^{-5} per milligram of protein in a 4-hour incubation period. Precursors present at concentrations of 0.1 mg./ml.

TABLE 4

DIFFERENCES IN SENSITIVITIES OF FORMATE INCORPORATION INTO THE GROSS PROTEIN FRACTION OF LEUKOCYTES TO CERTAIN CHEMOTHERAPEUTIC AGENTS*

	Normal	CLL	CGL	Acute
A-Methopterin	4.9 ± 2.2	6.0 ± 2.7	78.9 ± 1.0	44.6 ± 6.4
Chlorambucil	−88.8 ± 14.2	−141.0 ± 21.1	−13.1 ± 4.3	8.5 ± 7
Azaserine	40.6 ± 9.6	56.8 ± 3.9	52.3 ± 3.8	47 ± 10
Myleran	12.5 ± 2	−3.3 ± 5.9	10.2 ± 5.1	−2.8 ± 2
6-Mercaptopurine	13.0 ± 11.9	−1.0 ± 3.9	21.2 ± 4.7	8.8 ± 5.8
Purine riboside	79.6 ± 11	79.2 ± 10.9	96.4 ± 1.8	80.4 ± 3.5

* Figures represent per cent inhibition. Negative values represent per cent stimulation. Inhibitors present at a concentration of 100 μg./ml.

to some of the chemotherapeutic agents. TABLE 4 illustrates some examples of such differences in sensitivity.

These data demonstrate several quite, different types of response. A-Methopterin, an analogue of folic acid that is involved in metabolism of one carbon unit[4] has relatively little effect on the incorporation of formate into normal and CLL leukocytes, only a moderate effect on acute leukemia leukocytes, but a very marked inhibition on the incorporation of formate into the gross protein fraction of CGL leukocytes.[1, 2] This differential effect is also shown by certain other folic acid antagonists such as pyrimethamine (Daraprim)[4] and triazine.[4] FIGURE 1 strikingly illustrates a dose-response curve for the effect of Daraprim on the incorporation of formate into the gross protein fraction of CGL and CLL cells.

Chlorambucil, a drug used for therapy of Hodgkin's disease and for chronic lymphatic leukemia,[4] has a very different effect. This agent has minimal effects on CGL cells, but markedly stimulates the incorporation of formate into normal cells and, to an even greater degree, into CLL cells. The dose-response curve in FIGURE 2 shows the effects of chlorambucil on CLL and CGL cells.

Azaserine, which is usually considered an analogue of glutamine, inhibits the incorporation of formate into all cell types, but shows somewhat greater effects on CLL cells than on other cell types. The effects of azaserine are more difficult to evaluate than are those of the other drugs employed because of the pronounced time lag required before its effects become maximal.

Most of the agents that we have used do not, at any reasonable concentration, significantly affect the incorporation of any of the isotopic precursors that we have studied into the gross protein fraction. TABLE 4 shows this for

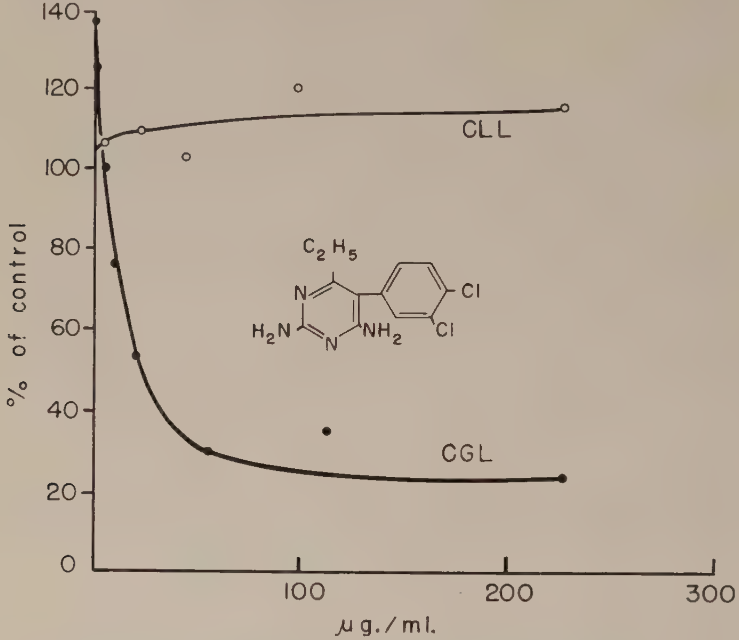

FIGURE 1. Dose response curves for pyrimethamine (Daraprim).

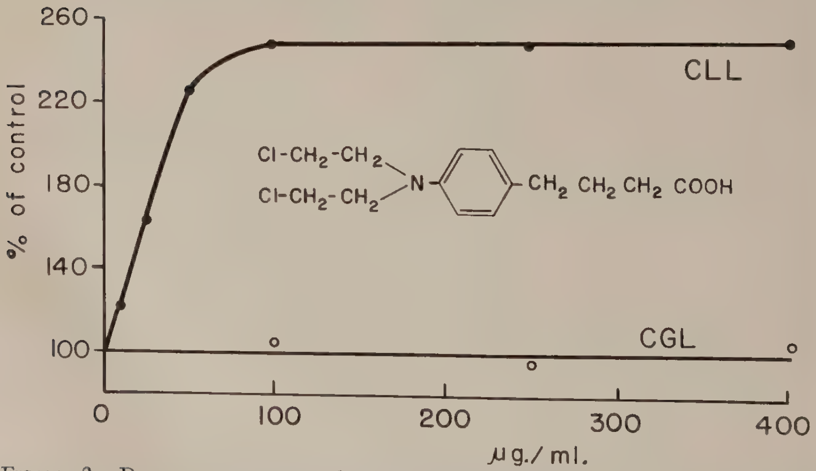

FIGURE 2. Dose response curves for the effect of chlorambucil on chronic lymphatic leukemia (CLL) and chronic granulocytic leukemia (CGL) cells.

the specific cases of busulfan (Myleran) and 6-mercaptopurine on uptake of formate. These two drugs have shown no effect on the incorporation of any of the isotopic precursors that we have used, although they are effective and widely used agents for chemotherapy of granulocytic and acute leukemia, respectively. Some agents, such as purine riboside, shown in TABLE 4, markedly affect all cell types at the levels tested.

Thus far we have considered only the incorporation of isotopic precursors into the gross protein fraction of cells. This is a relatively crude determination, since the isotope is distributed among several components of the cells. A much more detailed investigation of the distribution of the isotopic precursors into the various components of the cells is required for any insight into and understanding of the metabolic differences that have been mentioned. This type of work represents a challenging technical problem in view of the limited amounts of material available. However, we have made some progress in this area, and have uncovered some additional interesting metabolic differences between leukocytes of different types. I shall illustrate such differences, using the incorporation of C^{14}-formate.

When C^{14}-formate is metabolized by mammalian tissues it is distributed rather selectively to specific cellular components. These components and the location of the carbon derived from radioactive formate are shown in FIGURE 3.

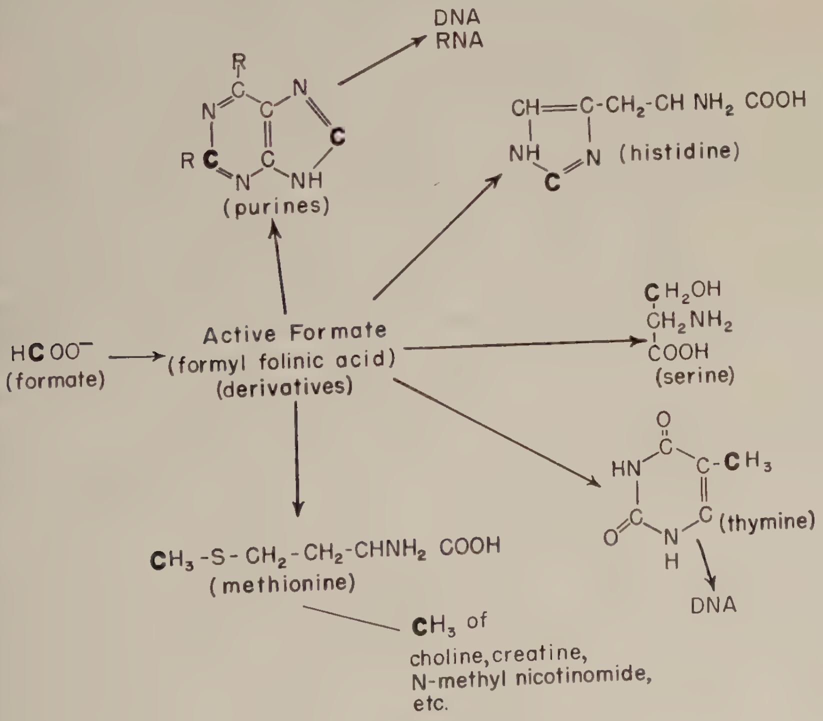

FIGURE 3

TABLE 5

RADIOACTIVITY DISTRIBUTION OF C^{14} FROM FORMATE-C^{14} INTO DIFFERENT CELL COMPONENTS: EFFECT OF A-METHOPTERIN

	CGL		CLL	
	RSA*	% Inhib.†	RSA*	% Inhib.†
RNA adenine	18.1	42.9	89.5	−33
RNA guanine	15.2	42.5	29.3	−80
DNA adenine	0.5	—	0.1	—
DNA guanine	0.5	—	1.1	—
DNA thymine	176.0	99.7	0.4	—
PB serine	21.4	55	53.0	5

* $\text{RSA} = \frac{\text{cpm}/\mu\text{M compound}}{\text{cpm}/\mu\text{M formate}} \times 10{,}000.$

† Per cent inhibition by A-Methopterin (10 μg./ml.).

We have isolated some of these components from CLL and CGL cells incubated with formate-C^{14}, using standard techniques of ionophoresis, ion exchange chromatography, and paper chromatography, and repurifying each component until the compound maintained a constant specific activity in successive purification steps. Some of the pertinent data are given in TABLE 5, which shows the specific activities of the major radioactive components in two typical experiments with CLL and CGL cells incubated with C^{14}-formate, the percentage of the total uptake accounted for by each fraction, and the effect of A-Methopterin on the specific activity of each component.

Several interesting observations are apparent from this chart. Thus, under identical conditions, the incorporation of formate into the ribonucleic acid (RNA) purines of CLL cells was appreciably greater than its incorporation into the comparable components of CGL cells. Incorporation into the desoxyribonucleic acid (DNA) purines was very slight in both cases; the apparent incorporation here may well be due to contamination of the DNA with a small amount of the more active RNA. The most surprising finding, however, is the very high specific activity of the DNA thymine in CGL cells and in the striking difference between CLL and CGL cells with respect to the incorporation of formate into thymine. These differences in thymine have been observed consistently.

TABLE 5 also shows that incorporation of formate into the purines and serine of CGL cells is much less sensitive to A-Methopterin than is its incorporation into thymine. It is evident, too, that the failure of A-Methopterin to inhibit the incorporation of formate into the gross protein fraction is apparent in the various components of the CLL cells, and in this experiment an actual stimulation of incorporation into the RNA purines of CLL cells was observed.

A particularly interesting difference between cells of different types has followed from the observations in TABLE 5. Thymine-2-C^{14}, incubated with cells of different types, is incorporated (probably intact into the DNA) at very slow but reproducible rates; data showing this are given in TABLE 6. A marked difference is seen between incorporation into CGL and CLL cells, the former

TABLE 6

INCORPORATION OF THYMINE-2-C^{14} INTO LEUKOCYTES

	*	Percentage of inhibition by A-Methopterin†
Normal	0.12	0
CLL	0.10	−10
CGL	1.44	−105
Bone marrow	3.74	−120

* μM incorporated/mg. GPF × 10^5 in 4-hour incubation period.
† 100 μg./ml.

incorporating the precursor much more rapidly than the latter. The effect of A-Methopterin is to stimulate thymine incorporation markedly into the CGL cells (as well as into normal bone marrow cells similarly incubated) while having no effect on incorporation into CLL or normal leukocytes. These observations suggest that A-Methopterin inhibits *de novo* thymine synthesis and thus increases thymine incorporation into the active thymine pool of CGL cells and thus into DNA. CLL and normal cells do not appear to synthesize or turn over DNA thymine appreciably, and so the A-Methopterin stimulation is not manifest.

These are some of the metabolic differences that we have observed between the metabolism of circulating human leukocytes of different types.

The fundamental or applied significance of these differences is not apparent, at least to our group. From a practical point of view it has not been possible thus far to draw any illuminating correlations between *in vitro* effects of chemotherapeutic agents and their clinical effectiveness.

From a fundamental standpoint, the data presented raise some puzzling biochemical questions. What does the large incorporation of formate into DNA thymine of CGL cells mean in terms of the assumed metabolic stability of DNA in nonproliferating cells? Does the great A-Methopterin sensitivity of formate incorporation into the methyl group of thymine suggest that folic acid derivatives are involved in carbon metabolism at the oxidation level of methyl alcohol? Does the incorporation that we have noted represent the *de novo* synthesis of new protein and nucleic acid from the precursors, or does it represent some sort of exchange reaction with preformed purines, amino acids, proteins, or nucleic acids? Much work will be required to answer these and other related questions.

Acknowledgments

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FEEDBACK MECHANISMS INFLUENCING PURINE RIBOTIDE SYNTHESIS*

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INTRODUCTION

The reactions involved in *de novo* synthesis of purine ribotides from low molecular weight precursors are now well known. The multienzyme system capable of effecting synthesis of purine ribotides from simple dietary precursors has been studied chiefly in the nonparticulate portion of pigeon liver homogenates.[3, 4] In this system a series of a dozen or more reactions takes place in the process of converting ribose-5-phosphate, glutamine, glycine, and other precursors into inosinic acid, the parent purine compound.[5-12] Although the intimate details of many individual reactions have now been elucidated, less attention has been given to the factors influencing or controlling the over-all rate behavior of the entire sequence.

Undoubtedly a number of factors influence the synthetic activity of this sequence. The usual ones of substrate and cofactor availability, enzyme activities, and energy sources have been considered to some extent in a previous discussion by Goldthwait.[13] In a multireaction sequence one might anticipate that a rate-limiting step might govern the maximal rate of the entire series of reactions, but no such rate-limiting step has been identified in the *de novo* synthesis of purines. It has been suggested that purine ribotide synthesis is under regulation by feedback control.[14, 15] Several feedback-control mechanisms for the regulation of biosynthetic processes have recently been described in microorganisms.[14-19] In at least two instances, a specific inhibition has been exhibited by an end product upon an enzyme concerned with its own production.[14, 16] In both cases the end-product inhibition operated at the level of comparatively remote precursors, thus suggesting an economical control mechanism whereby the biosynthesis of energetically expensive intermediates might be curtailed when sufficient end product was present.

One of the feedback controls mentioned above involved the regulation of *de novo* pyrimidine synthesis by the end product, cytidine monophosphate.[14] The existence of analogous feedback control mechanisms influencing purine biosynthesis is indicated by a number of observations. For example, the presence of unlabeled purines will suppress nucleic acid purine synthesis from labeled carbon dioxide[20, 21] or ammonium-N^{20} in bacteria, from glycine[22] in the rat, or from formate in bacteria[23] or intact rats.[24] Moreover, in certain purine-requiring mutants the presence of purine bases or compounds will prevent or suppress the accumulation of purine precursors at the stage of genetically impaired reactions.[15, 18] Also, concomitant oral administration of 5-amino-4-imidazolecarboxamide (AIC) significantly depresses the utilization of glycine-

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N^{15} for uric acid synthesis in normal man.[25] In none of these instances, however, has a precise localization of the control mechanism been demonstrated.

This paper deals with studies of mechanisms of feedback control of *de novo* purine synthesis that are operative in soluble supernatant solutions of pigeon liver homogenates. These extracts are sufficiently complex to contain the multienzyme systems capable of catalyzing the formation of the ribotides of the four major purine bases from simple substrates, yet sufficiently fractionated to avoid *de novo* synthesis or major destruction of glycine.[3] This latter point is of particular importance in studies such as this, in which labeled glycine serves as the tracer.

In the present study the effects of purine compounds upon *de novo* synthesis of inosinic acid (IMP) were evaluated initially. Several compounds were found that inhibited IMP synthesis and thus were of potential interest as possible participants in negative feedback control mechanisms. These included AIC and its riboside and ribotide, hypoxanthine, inosine, IMP and its higher phosphates, adenine, xanthine, and guanine. For various reasons these compounds did not appear to be the primary regulators. In the crude supernatant adenosine-5′-monophosphate (AMP) and guanosine-5′-monophosphate (GMP) were not inhibitory. However, in a partially purified system, adenyl nucleotides were found to inhibit an early step in purine synthesis. These inhibitions appear to be of considerable potential interest as a feedback control. This paper contains a description of these studies and a determination of specific loci of action of inhibiting compounds. The more intimate details of the mechanisms involved are the subject of continuing investigations.

Material and Methods

Enzyme preparations. The soluble supernatant solution obtained by centrifugation of a 1:2 pigeon liver homogenate for 30 min. at 105,000 g following the procedure of Schulman *et al.*[3] was employed in initial experiments. The glycineamide ribotide synthesizing enzyme was prepared from pigeon liver acetone powder by the method of Goldthwait *et al.*[5] This preparation was employed in preparation of glycineamide ribotide (GAR)-C^{14} and also in studies of effect of inhibitors on the rate of GAR synthesis. For these studies an adenosine triphosphate (ATP)-regenerating system was prepared from rabbit muscle by the method of Ratner and Pappas.[26] Orotidine-5′-monophosphate pyrophosphorylase and decarboxylase were prepared from yeast following the procedure of Lieberman *et al.*[27] Glutamic dehydrogenase was dialyzed against 0.05 M phosphate buffer *p*H 7.4 until free of ammonium sulfate.

Materials. The materials used included glycine-1-C^{14}; GAR-1-C* prepared enzymatically from glycine-1-C^{14} by the technique of Goldthwait *et al.*,[5] and assayed as suggested by Hartman *et al.*;[6] adenine-8-C^{14}; and AIC-ribotide (AICRMP), which was a gift of Thayer French and J. M. Buchanan of Massachusetts Institute of Technology, Cambridge, Mass. The AICRMP was purified or, in the case of the barium salt, converted to acid form by chromatography

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* Goldthwait *et al.* have shown that the GAR-C^{14} formed with this dialyzed and Dowex-1-treated acetone powder extract in the absence of formate is radiochemically about 93 per cent pure.

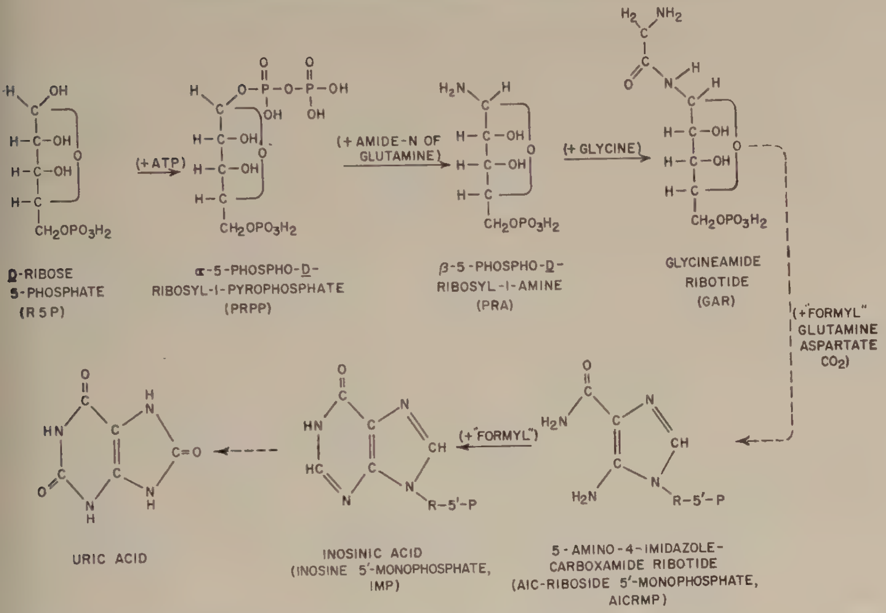
D-RIBOSE 5-PHOSPHATE (R5P)
(+ATP)
α-5-PHOSPHO-D-RIBOSYL-1-PYROPHOSPHATE (PRPP)
(+AMIDE-N OF GLUTAMINE)
β-5-PHOSPHO-D-RIBOSYL-1-AMINE (PRA)
(+GLYCINE)
GLYCINEAMIDE RIBOTIDE (GAR)
(+"FORMYL" GLUTAMINE ASPARTATE CO2)
5-AMINO-4-IMIDAZOLE-CARBOXAMIDE RIBOTIDE (AIC-RIBOSIDE 5'-MONOPHOSPHATE, AICRMP)
(+"FORMYL")
INOSINIC ACID (INOSINE 5'-MONOPHOSPHATE, IMP)
URIC ACID

FIGURE 1. Biosynthesis of inosinic acid (IMP). Only those substrates and intermediates of particular interest for the present study are shown.

on a Dowex-1-Cl⁻ column.[28] Barium ribose-5-phosphate was converted to the potassium salt by passage through a Dowex-50-K column. The α′-5-phosphoribosyl-1-pyrophosphate (PRPP) was prepared initially by the method of Kornberg *et al.*[29] and subsequently according to that of Flaks *et al.*[28] The 3-acetyl pyridyl diphosphopyridine nucleotide was obtained through commercial channels.

Following incubations, hypoxanthine was isolated as its silver picrate by the method of Schulman *et al.*[3] All counting was done in a Robinson gas-flow counter[30]* having an efficiency of 24 per cent when counting 3.3 mg. of material per 1.54 cm.^{2} planchet surface area, and was conducted to less than 5 per cent error.[31] Kinetic measurements of optical density changes were made in a Beckman DU ultraviolet spectrophotometer at room temperature in quartz cells having a light path of 1.0 cm.

RESULTS

Synthesis of IMP from Remote Precursors

FIGURE 1 summarizes the compounds involved in *de novo* synthesis of IMP with which we are concerned in this study. In experiments in which the overall syntheses of IMP from glycine + R-5-P, glycine + PRPP, or glycineamide ribotide, were compared (TABLE 1), there was a somewhat more efficient synthesis of IMP as more proximal precursors were employed. However, no

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* Built by Robert Dooley, Biophysics Laboratory, Harvard Medical School, Boston, Mass.

TABLE 1

De Novo SYNTHESIS OF IMP FROM REMOTE PRECURSORS

Experiment	Glycine-1-C^{14}		Glycineamide-ribotide-1-C^{14} (.25 µmole)
	R-5-P (7 µmoles)	PRPP (2.3 µmoles)	
	µmoles hypoxanthine synthesized		
1	0.43 0.39	0.60 0.83	
2		0.76 0.92	0.089 0.078
3	0.74	0.65 0.66	0.068 0.077

Each vessel contained 3.3 ml. of pigeon liver extract, 1:2 in 0.038 M phosphate buffer containing 0.13 M KCl, 0.01 M $MgCl_2$, and 0.043 M $KHCO_3$,[3] (30 mg. protein per ml. of buffer); 2 µmoles ATP; 15 µmoles K-3(−)-phosphoglycerate; 30 µmoles Na formate; 7.5 µmoles glutamine; and 7.5 µmoles Na aspartate. As indicated, some vessels also contained either 14 µmoles glycine-1-C^{14} plus 7 µmoles R-5-P or 2.3 µmoles PRPP, or 0.25 µmoles glycineamide-ribotide-1-C^{14} (specific activity of both tracer compounds was 10^4 cpm/µmole). Total volume 4.3 ml. Incubated 30 min. at 38°C. in air. Thereafter, 10 mg. of hypoxanthine was added as carrier, protein was precipitated with trichloroacetic acid, ribose compounds were hydrolyzed with HNO_3, and silver hypoxanthine picrate was isolated, recrystallized, and counted.[22]

clear-cut indication of an early rate-limiting step was obtained. Studies by Hartman *et al.*[4] employing labeled glycine, GAR, and formyl GAR have similarly not disclosed a rate-limiting step in IMP synthesis, although the reaction synthesizing PRPP seemed to be rate-limiting with respect to GAR synthesis under conditions of their experiments. The studies of Goldthwait *et al.*,[32] however, suggest that the reaction synthesizing β-5-phosphoribosyl-1-amine (PRA) may be the slow step. More detailed studies will be required to settle these points, but present results do not show that the activity of any of these early steps is intrinsically rate-limiting.

Effect of Purine Compounds on de Novo Synthesis of IMP from Labeled Glycine

TABLE 2 shows the results of selected studies in which the effects of various purine bases, nucleosides, and nucleotides upon *de novo* synthesis of IMP were determined. In these studies ribose-5-phosphate (R-5-P) served as the source of the ribose moiety. Inhibitory effects were obtained with adenine, xanthine, AIC-riboside (AICR) and -ribotide (AICRMP), inosine, and inosine-5′-monophosphate. Lesser degrees of inhibition were observed with AIC, inosine-5′-diphosphate (IDP), and -triphosphate (ITP). A correlation of degree of inhibition with concentration of adenine is shown in FIGURE 2, where it is seen that greater than 90 per cent inhibition occurs at 10^{-3} M adenine.

When R-5-P was replaced by PRPP (TABLE 3), xanthine, inosine, and IMP were no longer inhibitory, whereas adenine and AICR retained their inhibitory actions. AIC gave variable results. The results of TABLE 3 suggested that xanthine and IMP acted by inhibiting synthesis of PRPP, and that the other

TABLE 2

INHIBITION OF IMP SYNTHESIS FROM GLYCINE AND R-5-P

Experiment	1	2	3	4	5	6	7	8
Addition	*μmoles of hypoxanthine synthesized*							
None	0.19	0.10	0.30	0.61	0.45	0.76	0.91	1.44
AIC		0.07			0.33		0.49	1.16
AICR					0.24		0.33	0.58
AICRMP								0.001*
Adenine	0.03							
Adenosine		0.11		0.42				
Desoxyadenosine					0.54			
AMP-5′		.09		0.64				
Hypoxanthine							0.90	
Inosine					0.23	0.45		
IMP-5′					0.17	0.39		
IDP					0.38			
ITP					0.35			
Xanthine			0.02	0.10				
Xanthosine		0.11						
XMP-2′ + 3′					0.73			
Guanine			0.42					
Guanosine		0.10						
Desoxyguanosine					0.68			
GMP-2′ + 3′		0.12		0.52				
GMP-5′					0.47			
DesoxyGMP-5′					0.47			
GTP		0.09						

Complete system as in TABLE 1, except that all vessels contained 14 μmoles glycine-1-C^{14}. In experiments 1–3, 14.1 μmoles R-5-P were added, in experiments 4–8, 3.5 μmoles R-5-P were added. Potential inhibitors 4 μmoles each. Total volume 4.4 ml. Incubated 30 to 40 min. at 38° C. in air. Control values in this and subsequent tables represent averages of duplicates; other values represent single vessels.

* If 5-amino-4-imidazolecarboxamide-riboside-5′-monophosphate (AICRMP) had not been inhibitory, its "dilution" effect would have yielded a value of 0.38 μmoles of IMP synthesized (as hypoxanthine).

compounds acted either by depleting the system of PRPP or by inhibiting at some point beyond PRPP utilization for purine synthesis.

Effect of inhibitory compounds on conversion of GAR to IMP. When glycine-1-C^{14} and PRPP were replaced by GAR-1-C^{14}, none of the inhibitory compounds interfered significantly with *de novo* synthesis of IMP (TABLE 4) despite their addition in concentrations 16 times that of GAR. The apparent abolition of *de novo* synthesis of IMP in the presence of AICRMP can be explained adequately as a trapping effect of the labeled intermediate by the relatively large pool of added AICRMP, which is an obligatory intermediate in IMP synthesis. It may be concluded that all of the compounds that exerted inhibitory effects did so prior to formation of GAR.

Effect of inhibitory compounds on GAR synthesis. In a study of GAR synthesis from glycine-1-C^{14}, glutamine, and ATP, in which the ribosyl donor was R-5-P, several compounds were inhibitory (TABLE 5). These included AIC, AICRMP, IMP, adenine, xanthine, hypoxanthine, and guanine. When PRPP was substituted for R-5-P in this system, only AICRMP was inhibitory

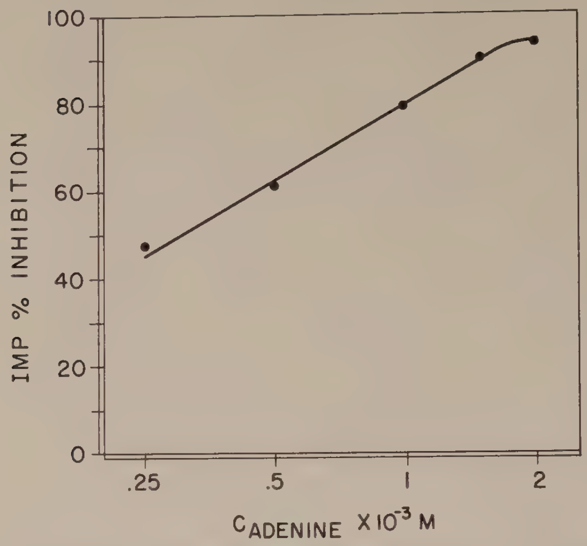

FIGURE 2. Inhibition of the biosynthesis of inosinic acid (IMP) by adenine. The relationship of adenine concentration to degree of inhibition of inosinic acid synthesis is shown.

TABLE 3
INHIBITION OF IMP SYNTHESIS FROM GLYCINE AND PRPP

Experiment	1	2	3
Addition	*μmoles of hypoxanthine synthesized*		
None	0.19	0.66	0.65
AIC	0.13		0.64
AICR	0.12		0.41
Inosine			0.62
IMP	0.21		0.71
Adenine	0.11	0.20	
Xanthine	0.19	0.66	0.56

Conditions as in TABLE 2, except that PRPP (about 40 per cent pure, remainder being largely R-5-P) was added as the ribosyl donor. Additions, 4 μmoles each.

to any appreciable extent. The results of TABLE 5 suggest that AICRMP interfered with synthesis of GAR beyond the stage of PRPP synthesis, and that the other compounds either interfered with PRPP synthesis or diverted PRPP from utilization for GAR synthesis into other fates.

Localization of Inhibitory Actions

Adenine, hypoxanthine, and guanine. The data of TABLE 6 show that the inhibitory effects of adenine in the system in which PRPP was employed were partially reversed at several concentration levels by additional PRPP. These results, as well as those reported above, suggested that adenine might have diverted PRPP from the main course of *de novo* synthesis. Subsequent studies demonstrated that PRPP was indeed being utilized by adenine to an appre-

TABLE 4

SYNTHESIS OF IMP FROM GAR-C^{14} IN PRESENCE OF INHIBITORS

Experiment	1	2
Addition	*μmoles of hypoxanthine synthesized*	
None	0.045	0.026
AIC	0.053	
AICR	0.041	
AICRMP	0.000	
IMP		0.027
Adenine	0.039	
Xanthine	0.058	

Same conditions as in TABLE 1, GAR experiments. Additions, 4 μmoles each.

TABLE 5

INHIBITION OF GAR SYNTHESIS

Ribosyl donor	R-5-P	PRPP
Addition	*μmoles of GAR formed*	
None	1.48	0.18
AIC	0.23	0.12
AICR		0.16
AICRMP-5′	0.20	0.08
Hypoxanthine	0.28	
IMP-5′	1.13	0.14
Adenine	0.21	
AMP-5′	1.46	
Xanthine	0.57	0.17
Guanine	0.50	

Each vessel contained 20 mg. of lyophylized powder of dialyzed Dowex-1-treated extract of pigeon liver acetone powder, 5 μmoles of glycine-1-C^{14}, 14 μmoles of Na 3(−)phosphoglyceric acid (3-PGA), 6.4 μmoles of $MgCl_2$, 0.06 mg. of lyophylized powder of rabbit muscle extract, and 25 μmoles of K_2HPO_4, *p*H 7.4. In the first experiment each vessel also contained 4 μmoles of KR-5-P, 2 μmoles of ATP, 4 μmoles of glutamine, 10 μmoles of glutathione. In the second experiment each vessel contained instead 0.4 μmoles of PRPP (40 per cent pure), 1.5 μmoles ATP, and 1 μmole glutamine. Total volume, 1.0 ml. in all cases. Incubated 20 min. at 38° C. in air, inactivated in boiling water bath, 1 min. GAR-C^{14} separated on Dowex-50-NH_4^+ columns[4] and assayed by C^{14} counting.

TABLE 6

EFFECTS OF PRPP ON ADENINE INHIBITION OF IMP SYNTHESIS

	Hypoxanthine synthesized		
PRPP (*μmoles*)	0.5	2.5	5.0
	μmoles		
Adenine (μmoles) 0	0.071	0.25	0.31
2	0.021	0.19	0.14
4	0.003		0.09

Conditions as in TABLE 1, except that PRPP was added in the amounts shown. Total volume, 4.0 ml.

Table 7
Utilization of PRPP

Addition	(1)	(2)
	µmole of PRPP utilized	
None	0.00	0.00
AIC	0.14	
AICRMP		0.00
Hypoxanthine	0.16	
IMP-5′		0.01
Adenine	0.24	
Xanthine		0.02
Orotic Acid	0.04	

Each vessel contained 20 mg. of lyophylized powder of dialyzed, Dowex-1-treated extract of pigeon liver acetone powder, 0.30 µmole of PRPP, 6.4 µmole $MgCl_2$, 25 µmoles KH_2PO_4, *p*H 7.4, and 4 µmoles base addition, where indicated, (except for AICRMP which was 2 µmoles). Total volume 0.6 ml. Incubated 20 min. at 37° C., inactivated in boiling water for 1 min. Treated with 30 mg. activated carbon (Darco)S-51, and filtered. Darco washed with 1 ml. cold water. Pooled filtrate analyzed for PRPP.

ciable extent (TABLE 7). Furthermore, when adenine-8-C^{14} was used, synthesis of AMP-C^{14} was demonstrated in this system, a result to be anticipated from the observation that pigeon liver contains an active adenylate pyrophosphorylase.[27, 33]

Certain other bases were also shown to utilize PRPP, though less extensively than adenine. AIC and hypoxanthine both utilized about one half of the available PRPP under conditions of the study shown in TABLE 7. Since guanine is converted to ribotide form by the same pyrophosphorylase that acts upon hypoxanthine,[34] PRPP utilization is also the probable mechanism by which guanine inhibited GAR synthesis. Hypoxanthine and guanine both utilize PRPP, and both inhibit GAR synthesis (TABLE 5), yet neither causes a decrease in apparent IMP-C^{14} accumulation in the complete system (TABLE 2). These differences in results are probably related to the quantities of R-5-P added. The inhibitory effects on GAR synthesis were observed in systems in which R-5-P was limiting.

It appears probable then that utilization of PRPP by free bases in the process of conversion to ribotide forms (FIGURE 3) results in a suppression of *de novo* ribotide synthesis from precursors of smaller molecular weight when PRPP is limiting. It is of interest that orotic acid was not inhibitory of IMP synthesis and also did not utilize PRPP in these preparations (TABLE 7).

Xanthine and IMP. In order to investigate the mechanism of inhibition caused by xanthine and IMP it was desirable to be able to follow PRPP synthesis from ATP and R-5-P kinetically so as to measure the influence of these inhibitors upon the rate of this reaction. It was found that the fraction of the soluble pigeon liver extract precipitated by ammonium sulfate between 20 and 40 per cent of saturation could be used after dialysis for such studies when coupled with orotic acid and the yeast "ethanol fraction" of Lieberman *et al.*,[27] which contains orotidylic pyrophosphorylase and decarboxylase. In this reaction, as indicated in FIGURE 4, the rate of synthesis of PRPP can be followed

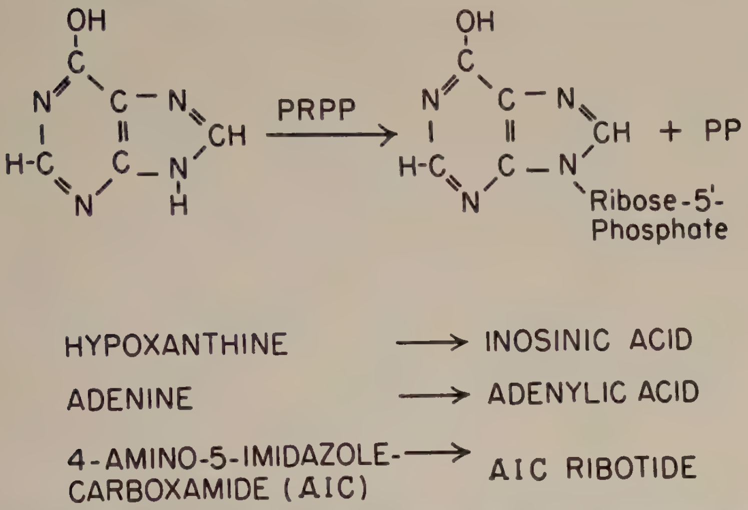
PRPP
+ PP
Ribose-5'-Phosphate
HYPOXANTHINE → INOSINIC ACID
ADENINE → ADENYLIC ACID
4-AMINO-5-IMIDAZOLE-CARBOXAMIDE (AIC) → AIC RIBOTIDE

FIGURE 3. Ribotide synthesis from bases, showing the reaction between the base and α-5-phosphoribosyl-1-pyropyrophosphate (PRPP). Two different phosphorylases are involved, one of which acts on 5-amino-4-imidazolecarboxamide (AIC) and adenine, the other on hypoxanthine and guanine.

spectrophotometrically by observing the decrease in optical density at 295 mμ. In the presence of an excess of either reactant the quantity of the other can be assayed with satisfactory accuracy. The Km for ATP was 1.4×10^{-3} M, and for R-5-P it was 2.9×10^{-5} M. A moderate inhibition of PRPP synthesis was observed at high levels of R-5-P (FIGURE 5), and this finding probably explains the better yields of IMP obtained at the lower level of R-5-P employed in the later experiments shown in TABLE 2.

When the effects of xanthine and IMP upon PRPP synthesis were examined in this system at levels of ATP and R-5-P ideal for demonstration of competitive events, it was found that neither compound was inhibitory (TABLE 8), even at concentrations equal to or greater than those of ATP and R-5-P. Apparently, therefore, the inhibitory effects observed in the cruder systems were due to extraneous influences and not directly to inhibition of the reaction that generates PRPP from ATP and R-5-P. We have not attempted to determine what these factors might be.

AIC compounds. In the system (TABLE 5) wherein synthesis of GAR-C^{14} from R-5-P or PRPP, glutamine, ATP, and glycine-1-C^{14} was studied, as well as in the complete system (TABLE 2), AICRMP was clearly inhibitory. AIC was also inhibitory, probably chiefly because of its utilization of PRPP; this mechanism was more effective in experiments in which PRPP was being synthesized than in those in which it was added in larger amount as a substrate (compare TABLES 2, 3, and 5). The product of the reaction of AIC and PRPP is AICRMP,[28] and in certain experiments (TABLE 5, center column) sufficient of this ribotide was probably synthesized to exert an additional effect. In these studies it appeared that the riboside may have been inhibitory, to some

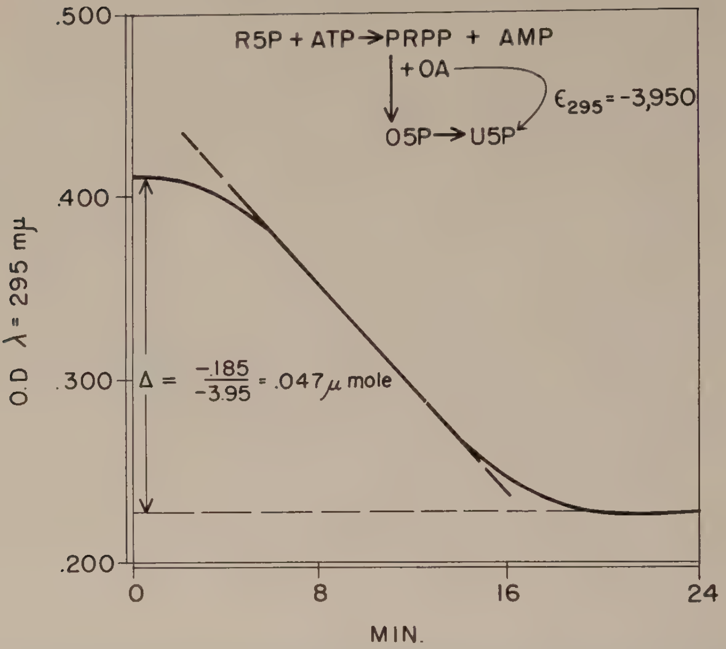

FIGURE 4. Spectrophotometric assay of PRPP synthesis. The formation of PRPP from R-5-P and ATP is measured kinetically by coupling the synthetic reaction with those that convert orotic acid to uridylic acid. The slope of the linear portion of the curve is a measure of the rate of the reaction. In the example shown 0.045 μmole of R-5-P was converted quantitatively to PRPP.

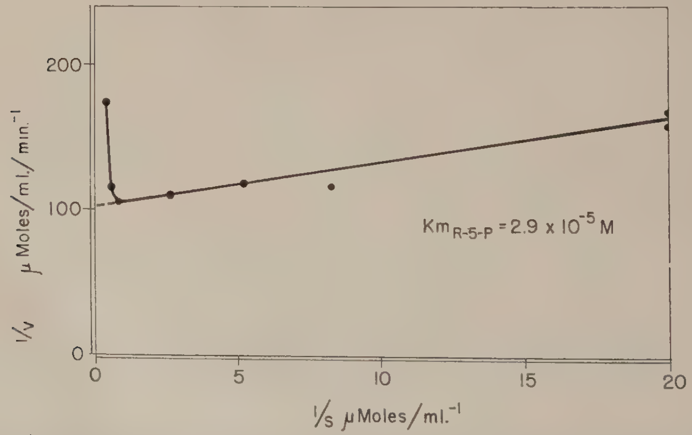

FIGURE 5. Double reciprocal plot of R-5-P concentration and reaction rate. The data are plotted according to Lineweaver and Burk.[35] Note the substrate inhibition at high concentrations of R-5-P.

TABLE 8
PRPP SYNTHESIS

Experiment	IMP	R-5-P	ATP	Rate of PRPP formation
	μmole/ml.	*μmole/ml.*	*μmole/ml.*	*mμmoles/10 min.*
1	0.00	0.045	1.6	28.4
	0.05	0.045	1.6	31.3
	0.25	0.045	1.6	33.4
2	0.00	0.97	0.5	38.8
	1.07	0.97	0.5	43.8
	Xanthine *μmole/ml.*			
3	0.00	0.045	1.6	26.6
	0.05	0.045	1.6	24.9
	0.25	0.045	1.6	26.0
4	0.00	0.97	0.5	34.9
	0.48	0.97	0.5	35.4

Km R-5-P = 2.5×10^{-5} M
Km ATP = 1.4×10^{-3} M

Each cell contained 50 μmoles of potassium phosphate buffer *p*H 8.1, the quantities of R-5-P, ATP, and IMP or xanthine listed above, 4 μmoles of $MgCl_2$, 0.2 μmoles of orotic acid, and 0.1 ml. of yeast "ethanol fraction" containing orotidylic pyrophosphorylase and decarboxylase, and 0.1 ml. of the PRPP synthesizing enzyme (see text) containing 0.2 mg. of protein, in a total volume of 1 ml. Light path 1.0 cm. Run in air at room temperature (25°C.), following the decrease in optical density at 295 mμ. The apparent Km for ATP may be high, since ATP-ases have not been ruled out.

extent in its own right (TABLES 2 and 3), or may have been converted to AICRMP by action of a nucleoside kinase, of the type acting upon adenosine.[36] It seemed possible that AICRMP inhibited one of the enzymatic steps concerned with GAR formation. Two (or possibly three) reactions are involved in this sequence: the formation of phosphoribosylamine (PRA), glutamate, and pyrophosphate from PRPP and glutamine;[7, 32] the probable formation of an "active glycine," suggested by an ATP requirement;[4, 7] and the coupling of PRA and glycine to form GAR.[7] Preliminary information has been obtained suggesting that the first of these reactions may be subject to feedback regulation.

Effect of ribotides upon glutamate production. We have evaluated the first of these reactions in preliminary studies wherein the generation of glutamate was used as a measure of its progress. The ammonium sulfate fraction precipitating between 20 to 40 per cent of saturation also contains an enzyme that generates glutamate from glutamine and PRPP and, by including glutamic dehydrogenase and 3-acetylpyridyldiphosphopyridine nucleotide in the reaction mixture, the progress of the reaction could be followed spectrophotometrically in terms of an increase of optical density at 363 mμ, representing generation of reduced coenzyme.[37] In this system, which has an absolute dependence on PRPP, azaserine is inhibitory, as anticipated,[38] but a high concentration is required, and its inhibition is easily overcome by glutamine (TABLE 9). AIC-riboside was without effect, whereas AIC-ribotide was inhibitory, but only at the 0.8 μmole/ml. level. IMP-5′ and GMP-5′ did not

TABLE 9

FORMATION OF GLUTAMIC ACID FROM PRPP AND GLUTAMINE

Experiment	Addition	Glutamine	PRPP	Rate of glutamate synthesis
	μmole/ml.	*μmole/ml*	*μmole/ml*	*mμmole/10 min./mg. of enzyme*
1	None	0.04	0.25	9.4
	Azaserine 10	0.04	0.25	3.6
	Azaserine 10	2.0	0.25	7.9
	AIC-riboside 2	0.04	0.25	11.0
	AIC-riboside 2	2.0	0.25	13.6
2	None	0.04	0.25	13.6
	AIC-ribotide 0.8	0.04	0.25	3.9
	AIC-ribotide 0.8	2.0	0.25	3.6
	AIC-ribotide 0.2	0.04	0.25	15.6
3	None	0.04	0.30	26.0
	GMP-5′ 2.0	0.04	0.30	37.0
	IMP-5′ 2.0	0.04	0.30	32.1
	AMP-5′ 2.0	0.04	0.30	9.4
	ATP 2.0	0.04	0.30	2.4
4	None	0.04	0.30	16.5
	AMP-5′ 2.0	0.04	0.30	2.9
	AMP-5′ 2.0	0.04	0.54	5.2
	ATP 2.0	0.04	0.30	4.5
	ATP 2.0	0.04	0.54	6.4

Each cell contained 50 μmoles of potassium phosphate buffer *p*H 8.1, glutamine, PRPP, and additions as indicated, 0.4 μmole of 3-acetylpyridyl diphosphopyridinenucleotide (APDPN), 3 μmoles of $MgCl_2$, and 0.1 ml. of glutamic dehydrogenase, diluted 1 to 10 and dialyzed versus 0.05 M phosphate, and 0.1 ml. of enzyme containing about 0.7 mg. of protein. Total volume 1 ml., light path 1.0 cm. Run in air at 25°C., following the increase in optical density at 363 mμ. A small endogenous reduction of APDPN is generally noted, and the reaction rate between 5 and 15 min. was recorded.

inhibit, but AMP-5′ and ATP were strongly inhibitory. The inhibitions by AICRMP, AMP, and ATP were not reversed by increasing the glutamine concentration, whereas those of AMP and ATP appear to be partially reversed by increasing the PRPP concentration. The present results therefore suggest that adenyl nucleotides may be competing with PRPP for binding by the PRPP synthesizing enzyme, and that the inhibitory effect of AIC ribotide may represent a less important effect manifested by a structurally related ribotide analogue. The enzyme system is currently being purified further to permit more careful kinetic studies and to ascertain whether or not the observed inhibitions are indeed primary effects upon the enzyme responsible for phosphoribosylamine generation.

Discussion

In the present study several purine end products have been shown to influence *de novo* synthesis of purine compounds. In the studies described it was doubtful that formation of enzymes[19] concerned with purine synthesis could have been a factor,[17] so that explanations were sought in terms of influences upon enzyme activities, or of competitive utilization of essential reactants. Although the data obtained initially in the unfractionated supernatant solution of the pigeon liver homogenate suggested that xanthine and nosinic acid (and other inosine compounds) might be interfering with synthesis of PRPP, a study of this reaction with a partially purified enzyme system disclosed no evidence for direct competition between xanthine or IMP and the primary reactants ATP and R-5-P. It was thus concluded that the effects observed in the cruder systems were extraneous ones, and that xanthine and IMP were not of genuine interest as feedback-regulating compounds, even though they produced undeniable inhibitions of synthesis of hypoxanthine compounds in the unfractionated supernatant, as initially reported for IMP by Buchanan and Schulman.[39] Since PRPP is necessary for pyrimidine ribotide synthesis,[27] for DPN synthesis[40, 41] and, possibly, for other reactions in addition to purine ribotide synthesis, there would have been little merit in a mechanism for control of PRPP synthesis based solely upon purine compounds.

The free bases, AIC, adenine, hypoxanthine, and guanine, were inhibitory of GAR synthesis by virtue of competition for PRPP and withdrawal of this essential reactant from the main sequence of purine synthesis. Inhibition of IMP synthesis was marked at high concentrations of adenine. Insofar as free bases are released by nucleoside phosphorolysis[42, 43] and salvaged by reconversion to ribotides,[28, 34, 40] they may act as inhibitors of *de novo* purine synthesis, providing that PRPP is limiting. In experiments in which bases were administered, and suppression of utilization of[20-25] or of formation[15] of another purine precursor demonstrated, this mechanism may well have been operative, as has also been suggested by Gots.[15] Active enzymes for converting bases to ribotides are found in liver,[28] in erythrocytes,[40] and, possibly, in other tissues. The equilibria of these reactions are far toward ribotide synthesis.[27, 28] In the synthesis of IMP from hypoxanthine and PRPP, no additional ATP is consumed, whereas in the *de novo* synthesis of IMP from PRPP and small molecular weight precursors, 5 moles of ATP are consumed, so that the salvage pathway permits a considerable economy of energy. The effectiveness of the salvage pathways will be influenced by the concentrations of free bases and by the activities of enzymes capable of oxidizing them. Since in most species xanthine oxidase appears to be limited to a certain few organs and is not demonstrable in others,[44] and since in higher forms adenase activity is not present,[45] it may be that reutilization mechanisms are of importance for some bases in certain tissues, and that under special circumstances free bases may be of interest as feedback regulators of *de novo* purine synthesis. It is doubtful, however, that they represent the major or delicate feedback regulators of *de novo* purine ribotide synthesis.

In order for a feedback regulation to operate effectively, it should consist of a competitive phenomenon involving an end product present in appreciable concentration within the cell and a substrate of an early step in the synthetic sequence by which this end product is generated, preferably of the first essentially irreversible step concerned specifically with this family of compounds.[14] From the standpoint of purine synthesis the step generating phosphoribosylamine from PRPP and glutamine is such a step,[7, 32] and a number of purine nucleotides might be logical regulators. In initial experiments it appeared that AIC-ribotide was the only compound whose point of action might be of potential interest. However, this compound is not really an end product, although it lacks only one carbon atom of forming IMP,[46] and is apparently a key compound in several bacterial reactions.[47, 48] Moreover, its concentration in the acid-soluble extract of pigeon livers is so low as to escape detection in the fractions eluted from a Dowex-1 formate column. Thus it was of considerable interest that the most recent results, obtained with a partially purified fraction of the pigeon liver supernatant, suggested that adenyl compounds might regulate the activity of this step, and that they might compete with PRPP in the reaction generating phosphoribosylamine. The failure to detect effects of adenyl nucleotides in the cruder system may have been due to various factors such as the low levels of ATP employed and the presence of an ATP-regenerating system that may have kept the AMP and ATP levels fairly constant in all flasks. The activity of AIC-ribotide may simply represent that of a structural analogue, and it is doubtful that in itself this compound is an important regulator of purine synthesis. Further work will be directed toward elucidating the mechanism of inhibition of this reaction by adenyl compounds, which has thus far been measured only in terms of glutamate production, and in determining whether the inhibitions described represent primary effects upon the theoretically vulnerable enzyme by which phosphoribosylamine is generated.

Summary

The subject of feedback regulation of purine ribotide synthesis has been investigated in pigeon liver extracts and partially purified enzyme systems. Several compounds were found to interfere with inosinic acid synthesis, all prior to formation of glycineamide ribotide. Free purine bases inhibited *de novo* purine synthesis by virtue of competition for phosphoribosylpyrophosphate; for various reasons this mechanism did not appear to be the process responsible for delicate regulation of purine ribotide synthesis. In a partially purified system 5-aminoimidazole-4-carboxamide ribotide, adenosine-5′-monophosphate, and adenosine triphosphate inhibited generation of glutamate from phosphoribosylpyrophosphate and glutamine. This inhibition of the step that is believed to generate phosphoribosylamine, the first purine-specific intermediate, is suggested as the site of a possible feedback control by adenyl nucleotides of the sequence of reactions responsible for purine ribotide synthesis. The enzyme forming glutamate from glutamine in the presence of PRPP has also been partially purified from extracts of acetone powder of human erythrocytes, and has been shown to be strongly inhibited by AMP-5′ and ATP, but not significantly by IMP-5′ or GMP-5′.

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DESOXYRIBONUCLEASES AND DESOXYRIBONUCLEASE INHIBITOR OF HEMATOPOIETIC TISSUE: THE EFFECT OF RADIATION*

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In 1952 at a symposium on the effects of radiation on biological systems at Brookhaven National Laboratories, Upton, N. Y., we suggested[3] that the nuclear damage observed after large doses of radiation might be mediated through an enzyme system rather than by direct effect on the chromosomal desoxyribonucleic acid (DNA), as was generally thought. Specifically, we suggested that radiation might inactivate an inhibitor of the enzyme desoxyribonuclease (DNase), thus permitting autolysis to occur. The experiments that we report here were intended to test this hypothesis.

We subjected CF-1 albino mice 12 to 14 weeks of age and of both sexes to 780 r total-body irradiation. This dose is slightly above the LD_{100} in 20 days for our colony.† At intervals organs were collected and analyzed for acid and alkaline DNase activity,[4] for the inhibition of alkaline DNase activity[5] by our methyl green method,[6] and for DNA content by the diphenylamine reaction.[7] The last parameter measures the number of cells present in the tissue.[8] Some animals were injected intravenously 24 hours after irradiation with 15 to 20 million homologous bone marrow cells[9] obtained from the long bones of CF-1 mice of approximately the same age. FIGURE 1 shows the survival of 100 irradiated controls and 87 animals injected with bone marrow. All of the controls had died by the eighteenth day, whereas at that time more than 80 per cent of the bone marrow-injected animals survived. Other animals were treated by exteriorization of the spleen, which was shielded in a lead box during the total-body irradiation as described by Jacobson *et al.*,[10] except that we did not sacrifice any splenic vessels. Following irradiation the spleen was returned to the abdominal cavity; we had no irradiation mortality in such animals. FIGURE 2 shows the influence of the irradiation on the weight of the spleen and its cellularity. The solid lines depict the controls, the dotted lines the bone marrow-injected animals. While the spleen weight in the controls falls to 25 per cent of normal, the total DNA content falls to slightly less than 10 per cent. This reflects the profound loss of cellularity, leaving a relatively acellular shrunken spleen. Following bone marrow injection there is rapid recovery in both parameters to normal on about the seventh day, after which the spleen hypertrophies to approximately 150 per cent of normal. The correspondence of the DNA and weight curves after the sixth day indicates return to normal cellularity. The curves for the shielded spleens show similar but

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* The work reported in this paper was supported in part by a grant from the American Cancer Society, Inc., New York, N. Y. Abstracts of previously presented portions of this paper have been published elsewhere.[1, 2] A more detailed report is in preparation.

† We are indebted to E. L. Simmons, Argonne National Laboratory, Chicago, Ill., for information on his experience with the lethality of various doses of irradiation on CF-1 mice.

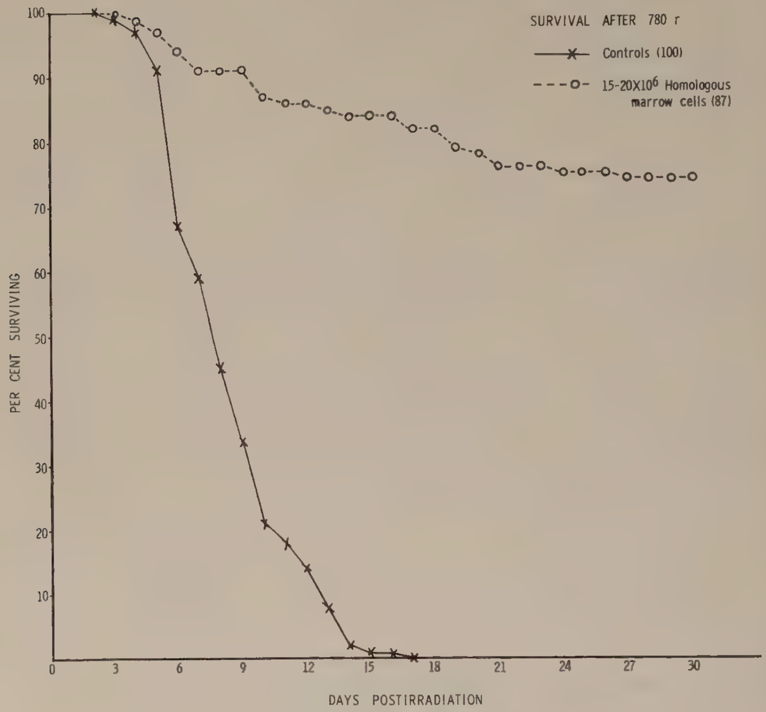

FIGURE 1. Survival after 780 r X irradiation.

somewhat less dramatic changes. In FIGURE 3 the influence of irradiation on the cellularity of the spleen is expressed in terms of DNA per mg. of spleen. It may be observed that simultaneously with the reduction in the cellularity of the organ, which occurs during the first 24 hours, there is a very marked rise in the acid DNase activity per average cell to approximately 700 per cent of normal. This activity then remains elevated for the duration of the life of the animal. A moderate fall in splenic acid DNase follows the peak in the control animals. We believe that this may be due to the death of the most severely damaged cells, which presumably had the highest DNase activity. Preliminary data indicate that the *alkaline* DNase activity follows a similar curve. The injection of bone marrow produces recovery of cellularity and return of the acid DNase activity to normal. This presumably reflects replacement of the damaged elements by cells that have normal acid DNase activity. The slight increase in acid DNase activity noted 30 days postirradiation may be attributed to the increase in splenic acid DNase associated with age.[2]

The findings in the spleen are consistent with the suggestion that irradiation destroys inhibitors of both acid and alkaline DNases, resulting in the autolysis that leads to cell death. Whereas there have been reports of the demonstration of an inhibitor of acid DNase in leukocytes,[11] in urine,[12] and in blood,[13] we have not yet succeeded in confirming its existence. However, we have

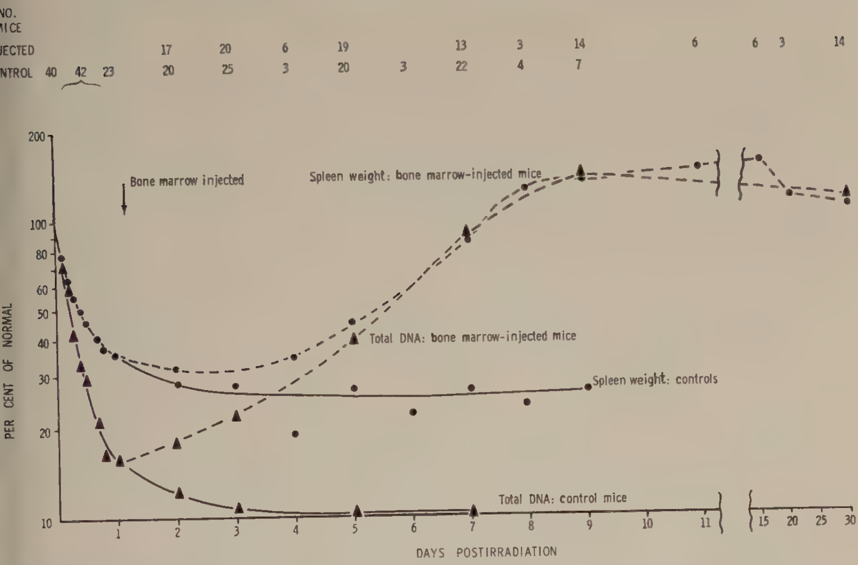

FIGURE 2. Weight and total DNA in spleen after 780 r X irradiation of control and bone marrow-injected mice.

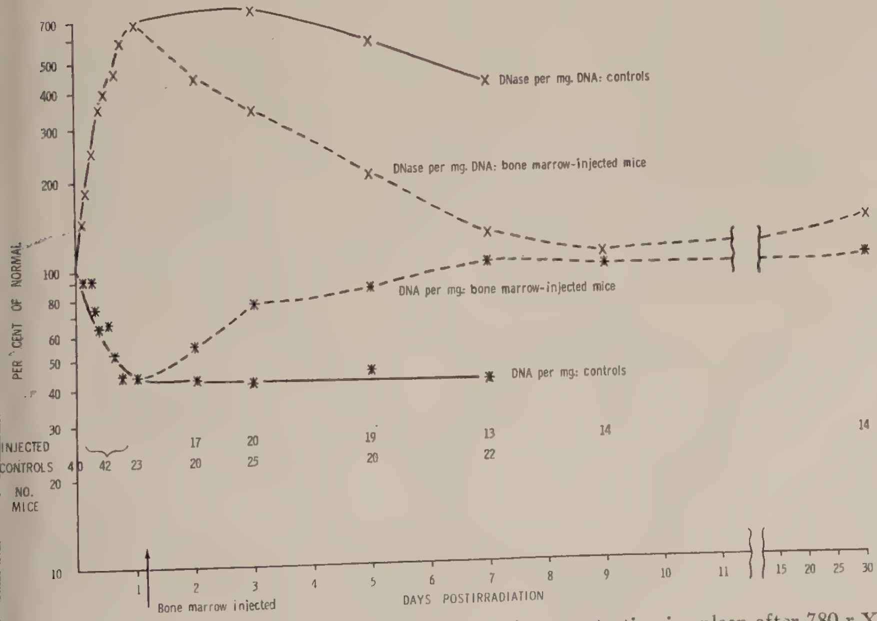

FIGURE 3. Acid DNase per average cell and DNA concentration in spleen after 780 r X irradiation of control and bone marrow-injected mice.

observed inactivation of the inhibitor of an alkaline DNase following irradiation (FIGURE 4), which is roughly reciprocal to the rise in spleen alkaline DNase activity. Whereas the alkaline DNase inhibitor activity remains at a minimum in the unprotected animal, bone marrow injection, as shown in FIGURE 5, results in a further drop followed by gradual return to normal. The further drop in inhibitor activity per average cell when the spleen is already becoming repopulated suggests repopulation with cells poor in inhibitor. Previous studies have demonstrated that lymphocytes are particularly abundant in inhibitor.[5] Evidence presented below on the slow repopulation of the irradiated thymus, as well as histological observations,[14, 15] suggest that lymphocytes repopulate the spleen less rapidly than myelopoietic and erythropoietic elements, which are poorer in alkaline DNase inhibitor. Thus, a return to normal of the inhibitor activity per average cell would await the slower repopulation by lymphocytes.

Changes in the liver are less dramatic than in the more radiosensitive spleen. FIGURE 6 shows that the unprotected irradiated animals suffer a loss of about one third of their hepatic cells. The animals that receive bone marrow 24 hours postirradiation show a similar loss in liver cell population, but recover by the eighth day. The acid DNase activity per average cell in the liver of

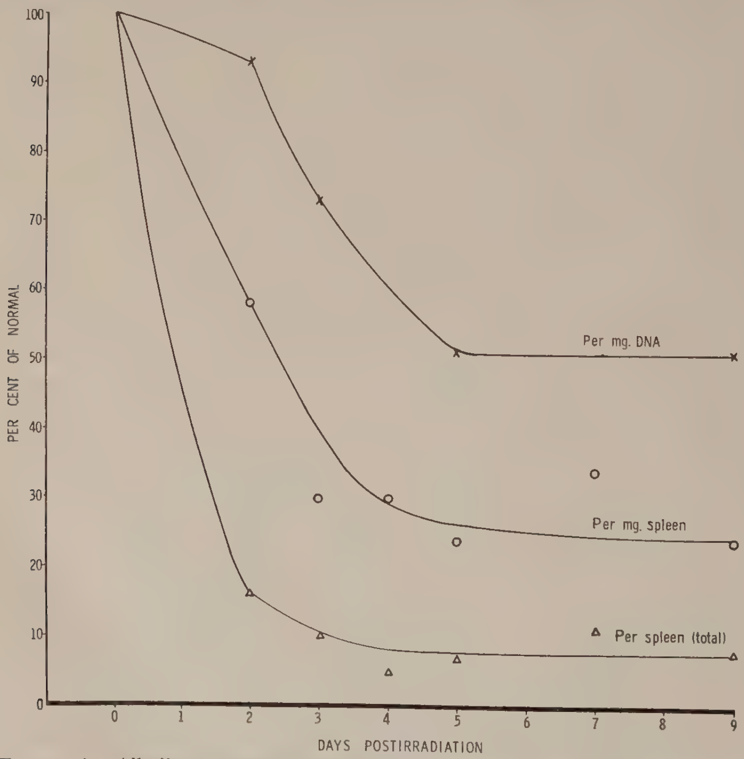

FIGURE 4. Alkaline DNase inhibitor in mouse spleen after 780 r X irradiation.

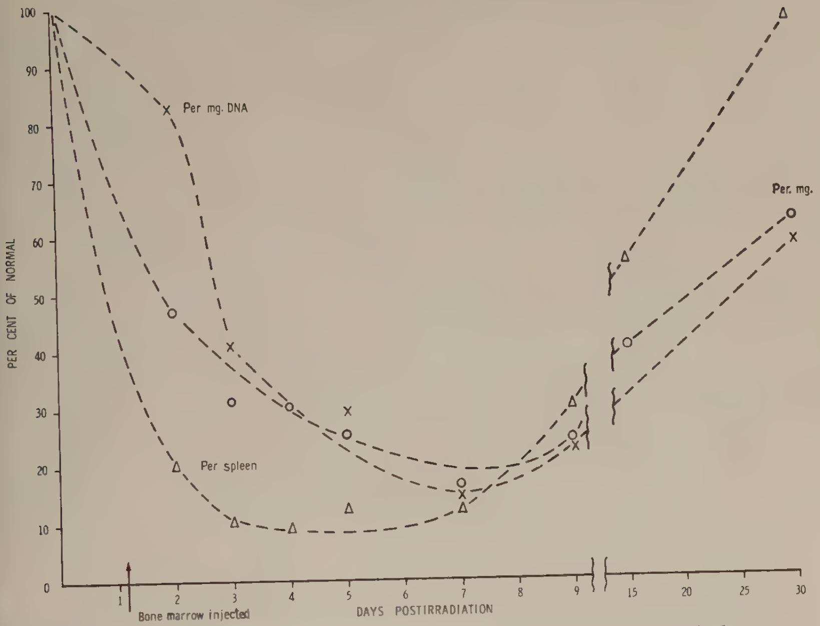

FIGURE 5. Alkaline DNase inhibitor in spleen after 780 r X irradiation in bone marrow-injected mice.

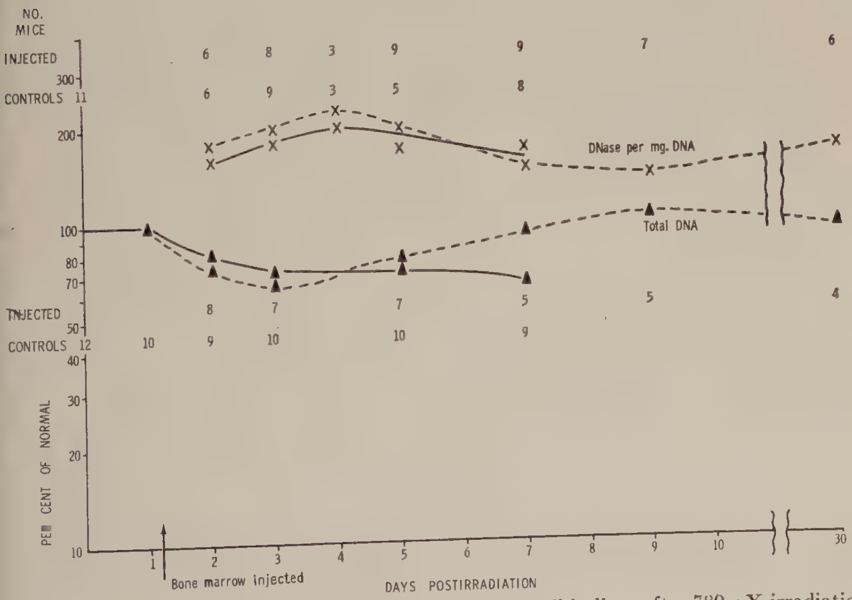

FIGURE 6. Total DNA and acid DNase per average cell in liver after 780 r X irradiation in control and bone marrow-injected mice. Broken line, injected animals; solid line, controls.

the irradiated animal rises to approximately 200 per cent of normal and remains at this level until death. In the spleen-shielded and bone marrow-injecte animals the increase in DNase activity per average cell is similar to that o the unprotected animal, and only partial recovery occurs. This is probably due to the fact that approximately two thirds of the cells remaining in the recovered animal are the original cells exposed to irradiation, unlike the situation in the spleen and bone marrow, where less than 10 per cent of the cells in the recovered organs are of the original irradiated population. Since the loss in cell number is small, the increase in total DNase in the liver is almost as great as the increase per average cell.

FIGURE 7 shows the course of events in the thymus of the irradiated control animal. Acid DNase activity follows a course very similar to that in the spleen, but it is even more dramatic, increasing to more than 2000 per cent in the average cell. The loss of cellularity is also more dramatic; the total number of cells decreases to less than 5 per cent of normal. As a consequence, the total DNase activity of the thymus shows only a relatively small rise to about 175 per cent as compared to the very marked rise in activity per average cell. As in the spleen, the weight curve reflects the cell loss without loss of

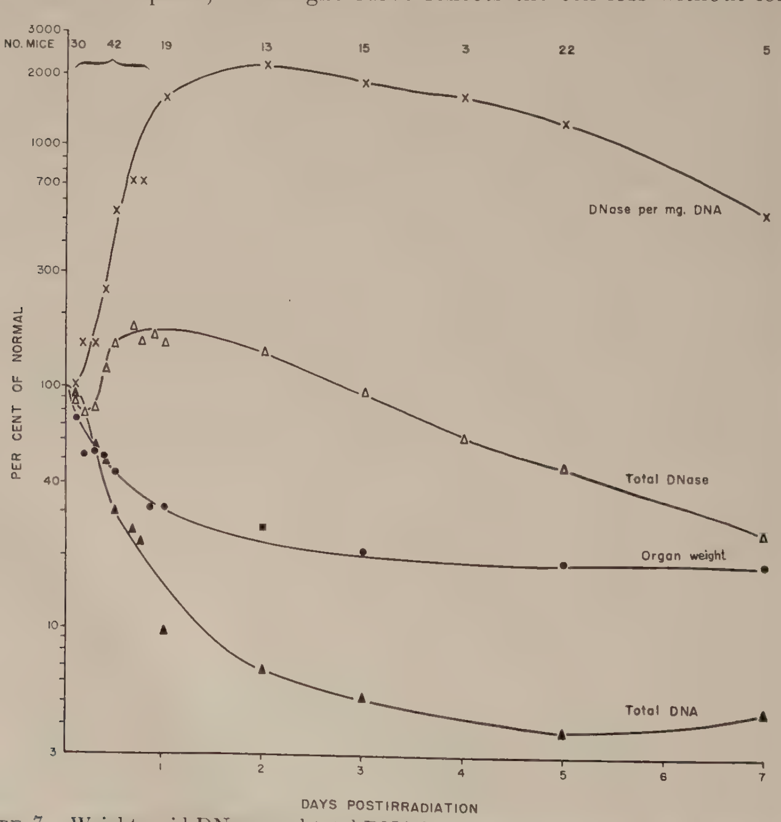

FIGURE 7. Weight, acid DNase, and total DNA in mouse thymus after 780 r X irradiation.

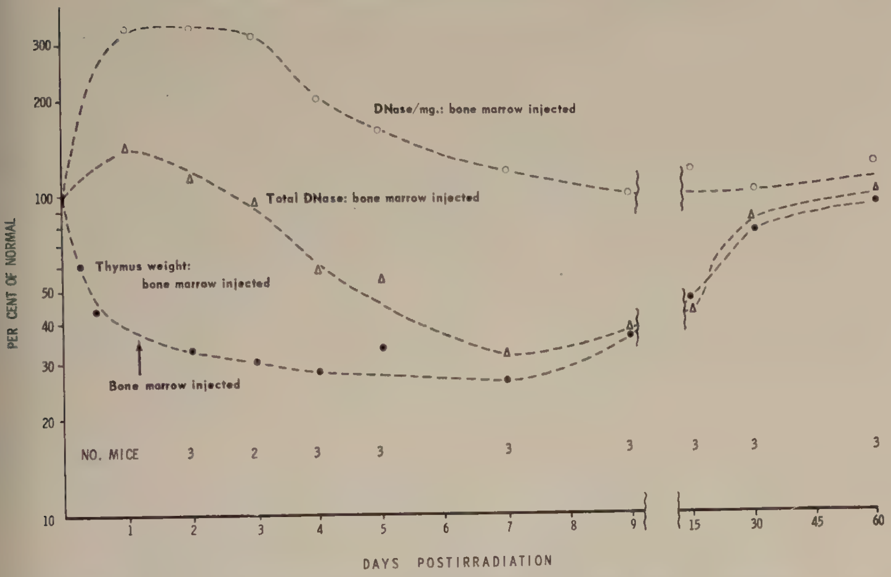

FIGURE 8. Acid DNase and weight of mouse thymus after 780 r X irradiation in bone marrow injected mice.

stroma, so that the reduction in weight is about 80 per cent, while there is a 95 per cent loss in cell number. The curves for the bone marrow-injected and the spleen-shielded animals (FIGURE 8) are almost identical to the controls for the first 7 days. Beginning at 7 days there is a slow return to normal. The values are all within 20 per cent of normal by the thirtieth day and are normal on the sixtieth day. The very slow recovery of the thymus as compared to that of the spleen indicates the much slower repopulation of lymphoid cells from the donated marrow than of the other hemic elements.[16]

FIGURE 9 indicates the logarithmic increase in acid DNase activity per average nucleated bone marrow cell following total-body X irradiation. On the seventh day the value is 2300 per cent of normal. Animals that received bone marrow intravenously on the first postirradiation day follow the same course as the controls for the first 3 days, then return to normal about the ninth day.

TABLE 1 presents the absolute data for the preirradiation organs and the maximal DNase values postirradiation. It may be noted that, although the thymus activity rises more than twentyfold and the spleen less than eightfold, the splenic DNase activity is still three times that of the thymus after irradiation.

In summary, our findings indicate that lethal total-body irradiation results in marked increase in the acid DNase activity of the average spleen, liver, thymus, and nucleated bone marrow cell of the mouse. The damaged cells show this effect maximally approximately 24 hours postirradiation except in the bone marrow, and they continue to demonstrate the abnormality for the duration of their survival. Bone marrow injection or spleen shielding results in repopulation of the hematopoietic organs by cells that have not been exposed to irradiation. These organs, therefore, show return to normal in acid DNase activity and in the inhibitor of alkaline DNase. The slower repopulation of the thymus compared to spleen and bone marrow is reflected in the slower return to normal of its DNase activity. In the liver, which is much less sensitive to irradiation, the increase in acid DNase activity per average cell is much less marked, and cell death is also much less extensive. However, the injury

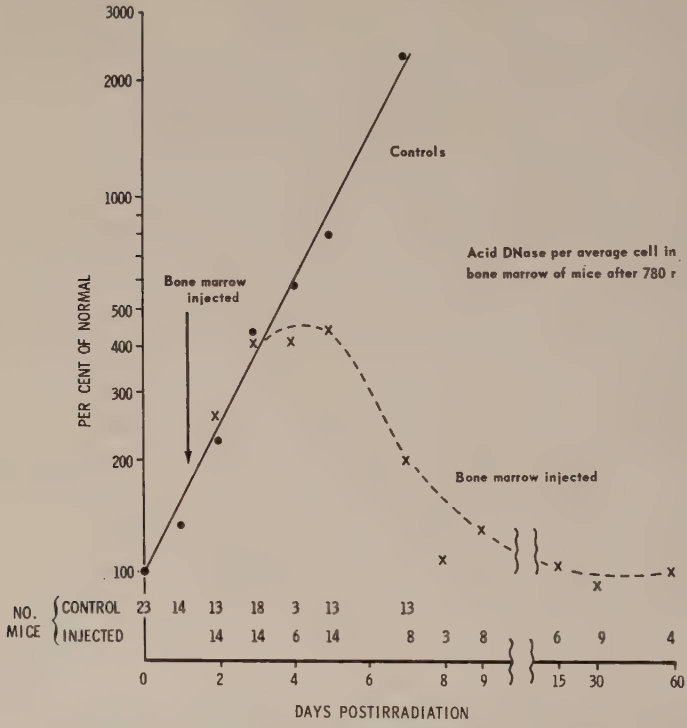

FIGURE 9. Acid DNase per average cell in bone marrow of mice after 780 r X irradiation

TABLE 1

	Spleen	Liver	Thymus	Bone marrow
Acid DNase units per 10^{-10} average cells* before X ray	32	3.9	3.8	27
Acid DNase units per 10^{-10} average cells max. post X ray	240	7.8	80	620
Organ weight before X ray, mg.	130	1500	56	—
Total mg. DNA-P before X ray	150	3000	100	—

* Based on 0.7×10^{-9} mg. DNA-P per average cell. See Kurnick[4, 6] for definition of DNase unit.

is a permanent one that may account, in part, for the reduced long-term survival of irradiated animals, even though they have been protected against short-term lethality by bone marrow injection or spleen shielding. The observations are consistent with, but do not verify, the hypothesis that irradiation injury may be due to inactivation or impaired synthesis of an inhibitor of acid as well as alkaline DNase, liberating the ubiquitous intracellular enzyme that causes the autolysis of the genic material and consequent cell death. Alternative hypotheses may be mentioned. Activation of DNase by direct X radiation effect appears improbable. It has been our experience that X rays inactivate crystalline bovine alkaline DNase *in vitro* (addition of protein to the solution protects the enzyme against inactivation, however). Preferential retention of the enzyme within the organ after its release from lysed cells has been suggested by Douglass and Day[17] and by Okada *et al.*,[13] who also observed increase in splenic acid DNase 24 hours postirradiation. However, a mechanism for the retention of a soluble enzyme for more than 7 days while tissue protein and DNA are extensively lost does not come readily to mind. Differences in X-ray sensitivity of cell types, with selective survival of cells rich in acid DNase, would also result in increased acid DNase activity per average cell. Whereas this effect may, in fact, be operative and account for part of the increase observed, it fails to account for the increases in total acid DNase activity in the organs, which are observed at least as transitory events before the cell numbers become extremely small. We consider, therefore, that inactivation of an inhibitor of acid DNase, analogous to that we have demonstrated for alkaline DNase, during or following radiation injury appears the most plausible hypothesis. We are continuing efforts to isolate and test directly for such an inhibitor of acid DNase.

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Part II. Enzymes in Red Blood Cells

RECENT ADVANCES IN KNOWLEDGE OF THE HEXOSE MONOPHOSPHATE SHUNT

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While many of the major advances in the field of enzymes in blood have been made by contributors to this publication, whose views I can present only at second hand, I feel that it would be useful, before discussing the work of our own laboratory, briefly to consider the over-all trends and lines of evidence relating to the pentose phosphate pathway of carbohydrate metabolism in animal tissues.

In an earlier publication of the Academy, Efraim Racker (1956) aptly referred to what might be called the revolution in our thoughts on carbohydrate metabolism brought about by the recognition of alternate pathways, these routes being superimposed on the familiar Embden-Meyerhof glycolytic sequence, and resulting in a more complex pattern that we were just beginning to learn to evaluate quantitatively. This situation still holds and, as usual, its analysis has involved a simultaneous development along a number of lines, the principal ones being first, enzymology and reaction mechanisms in purified systems, second, studies of the amounts and distribution in animal tissues of these enzymes and coenzymes at both the cellular and subcellular levels, and, third, evidence derived from the differential utilization of specifically C^{14}-labeled carbohydrates and intermediates.

The earlier and well-known work of Scott and Cohen and of Horecker *et al.* had clearly established by 1955 (for a review, see Dickens, 1955) the two steps of the enzymic oxidative pathway from glucose-6-phosphate (G-6-P) through the primary product 6-phosphogluconolactone and then 6-phosphogluconate (6-PG), followed by the oxidative decarboxylation of 6-PG by a second triphosphopyridine nucleotide (TPN)-linked dehydrogenase to give CO_2, derived from C-1 of glucose, together with D-ribulose 5-phosphate (Ru-5-P), as shown in FIGURE 1. The recent enzymologic developments have been concerned mainly with the elucidation of the subsequent fate of the pentose phosphate, especially the role of the two enzymes, transketolase and transaldolase, by Racker and Horecker and their colleagues (FIGURES 2 and 3).

Before these transformations can occur, a suitable mixture of pentose phosphate substrates must be provided. Two enzymes take part here: (1) pentose phosphate isomerase (PPI, more specifically designated as 5-phosphoribose isomerase) and (2) phosphoketopentose epimerase (PKPE), which might also be called 5-phosphoribulose-3-stereoisomerase, since it causes inversion at C-3 of the ketopentose:

$$\text{D-Ribose 5-phosphate} \underset{}{\overset{\text{PPI}}{\rightleftharpoons}} \text{D-ribulose 5-phosphate} \qquad (1)$$

$$\text{D-Ribulose 5-phosphate} \xrightleftharpoons{\text{PKPE}} \text{D-xylulose 5-phosphate} \qquad (2)$$

Reaction 1 was first described by Horecker *et al.* (1951) and accounts for the early observed formation of aldopentose phosphate (R-5-P) from the Ru-5-P resulting from the C-1 decarboxylation of G-6-P. The equilibrium value is subject to some uncertainty due partly to reaction 2, which occurs to a variable extent, values of ketopentose phosphate (KPP)/aldopentose phosphate (APP) ratio of about 0.3 having been reported earlier for enzyme preparations from yeast (at 25° C., Horecker *et al.*, 1951) and alfalfa (at 37° C., Axelrod and Jang, 1954). However, we found that the animal enzyme from red cells (Dickens and Williamson, 1955, 1956) gives reversibly a KPP/APP ratio of 0.5 at 37° C. This preparation was very weak in PKPE, yielding about 90 per cent pure D-ribulose 5-phosphate (figure 4, curve B): by contrast the similar preparation from skeletal muscle (figure 4, curve A, and figure 5) in addition to giving a rather higher ratio of 0.75 for KPP/APP, transformed about 55 per cent of the total KPP into D-xylulose-5-phosphate due to the presence of an active PKPE. This corresponds with a ratio Xu-5-P/Ru-5-P of 1.2 at 37° C. For the purified PPI-free bacterial epimerase from *Lactobacillus pentosus*, Hurwitz and Horecker have obtained the equilibrium ratio Xu-5-P/Ru-5-P = 1.5 at 25° C. With lysed and dialysed human red cells, however, Dische and Shigeura found the KPP/APP ratio to be about 1.7/1 at 33° C. The explanation of this remarkably high figure* involves the pre-

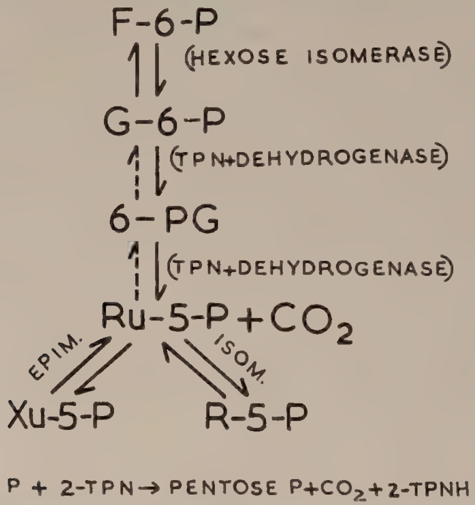


Figure 1. The hexose monophosphate (HMP) route of oxidative formation of pentose phosphates from hexose-6-phosphate (F-6-P). Abbreviations as in text. Also, epim. = phosphoketopentose epimerase (PKPE); isom. = pentose-phosphate isomerase (PPI). These reactions are the source of the R-5-P and Xu-5-P for stage 3, which is illustrated in figure 3.

* Values of Xu-5-P/Ru-5-P of 3 have since been reported by Tabachnik *et al.*

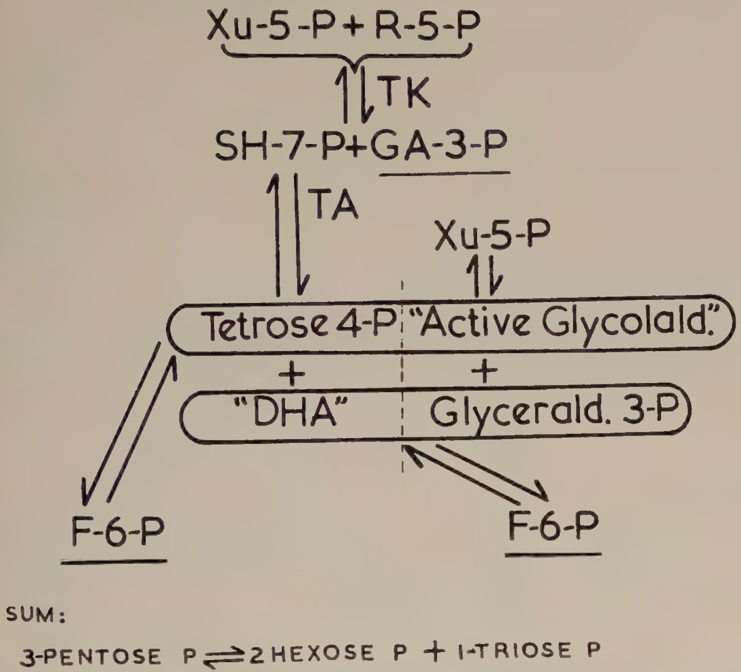


FIGURE 2. Hexose resynthesis from pentose phosphates by the consecutive action of transketolase (TK) and transaldolase (TA). After the formation of sedoheptulose-7-phosphate (SH-7-P) and triose phosphate by the action of TK on the pentose phosphates, TA splits the SH-7-P to tetrose phosphate, thus transferring "active dihydroxyacetone" ("DHA") to a molecule of glyceraldehyde phosphate, producing one molecule of fructose-6-phosphate (F-6-P). A second F-6-P molecule arises through the transketolation of "active glycoaldehyde" from xylulose-5-phosphate to the tetrose phosphate.

sumed presence of esters other than Ru-5-P and Xu-5-P in the ketopentose fraction.

The first recognition of a xylulose phosphate in the animal metabolism was by Ashwell and Hickman (1954, 1955, and 1957) who in 1957 partially purified the epimerase from calf spleen and further studied this reaction. These investigators find evidence of formation of an *erythro*-3-ketopentose phosphate in small quantities (our similar experiments indicated 1.3 per cent of the total KPP; Dische and Shigeura report less than 3 per cent of the total pentose phosphate). If this is not an artifact, it suggests the possibility of epimerization via the 2:3-*enediol* form of ketopentose phosphate, as is discussed both by Ashwell and Hickman (1957) and by Dische and Shigeura. Thus far, a coenzyme requirement (for example, for uridine compounds) has not been observed with these enzymes. An epimerase acting at C-4 of the pentose chain and converting L-ribulose-5-phosphate into D-xylulose-5-phosphate exists in *Lactobacillus pentosus*, and this appears to be the route by which this organism metabolizes L-arabinose (Simpson and Wood, Burma and Horecker). The

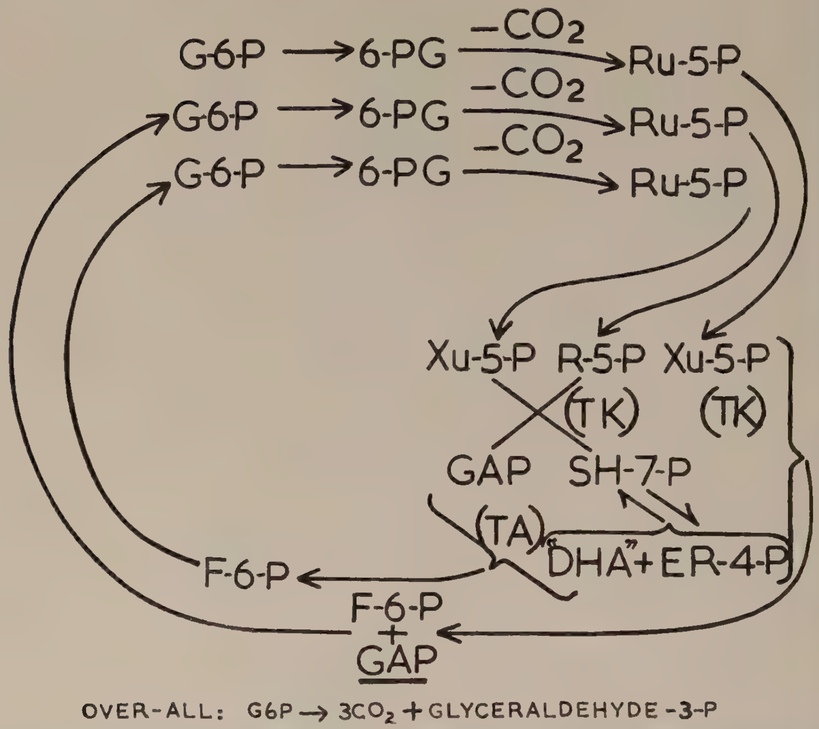


FIGURE 3. The complete HMP oxidative cycle. Since each of the two oxidative steps leading to Ru-6-P is coupled with the reduction of one mole of TPN^+, 6 moles of reduced TPN, requiring 3 O_2 for oxidation, are produced in the above cycle, with the effect of combustion of one triose unit to 3 CO_2 and 3 H_2O. The remaining glyceraldehyde-3-phosphate (GAP) molecule can be utilized for the synthesis of hexose phosphate by the action of triose phosphate isomerase, aldolase, and fructose diphosphatase, again giving F-6-P.

purification of a similar enzyme from *Aerobacter aerogenes* has been described by Wolin *et al.*

In blood serum, the formation of ketopentose from R-5-P indicates the presence of a PPI system. The activity varies greatly in sera from different species, but is only about one thousandth of that of the corpuscles (Bruns, 1956, 1957). However, its presence in serum apparently is not due to hemolysis. Considerably increased amounts are reported by Bruns in cases of liver damage, nephritis, and lymphosarcoma (FIGURE 6). The loss of orcinol-reacting pentose when R-5-P is incubated with human serum, reported by Mello, may be due in part to this effect, since in work undescribed in print done with Mello in our laboratory we have confirmed Bruns' observation. We also find little further transformation to sedoheptulose phosphate that would indicate weak PKPE and/or transketolase activity. Sedoheptulose formation in blood has been studied by Šonka and Palek in various pathological states.

At this stage we may also briefly note the occurrence in human serum of dehydrogenases for G-6-P and 6-PG reported by Wolfson and Willams-Ash-

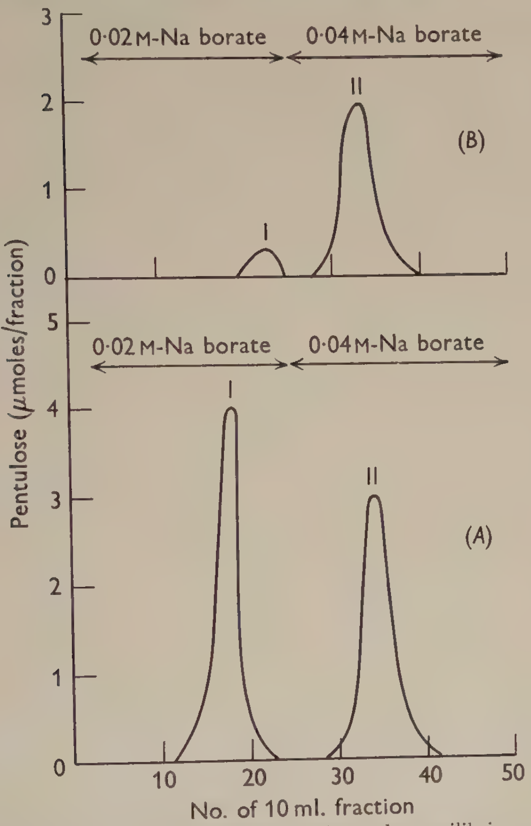


FIGURE 4. The separation of free ketopentose from the equilibrium mixture produced by enzyme preparations from skeletal muscle (*A*) and red blood cells of the horse (*B*) acting on ribose-5-phosphate. The ketopentose phosphates (KPP) were first separated from the aldopentose phosphates by column elution. After the hydrolysis of the phosphate esters by acid phosphatase the KPP fraction was adsorbed on Dowex-1 (borate form), and the free sugars were eluted as shown. Peak I = D-xylulose; Peak II = D-ribulose. Reproduced from Dickens and Williamson (1956) with the permission of the *Biochemical Journal*.

man. These TPN-linked enzymes are quite active, of course, in red cells, in which material they (and the TPN with which they react) were originally discovered by Warburg (see Warburg *et al.*). On the other hand, red cells are usually weak in aldolase, which in serum has similar activity (moles substrate/ml./hr.) to 6-PG dehydrogenase. The pentose phosphate isomerase activity of serum is much higher, comparable with that of hexosephosphate isomerase and lactic dehydrogenase. These points are discussed fully elsewhere in this monograph.

The Transketolase-Transaldolase Sequence

Since the earlier preparations designated as Ru-5-P are now seen to have been contaminated to a varying extent with Xu-5-P and as, also, the preparations of PPI certainly contained PKPE, the earlier view that Ru-5-P was the substrate of transketolase (TK) must be revised and, in fact, is now known to be incorrect. It was shown almost simultaneously by Racker and his colleagues (Srere *et al.*) and by Horecker *et al.* (1956) that PKPE as well as PPI must have been present for TK to act on R-5-P, thereby producing a reaction mixture consisting of R-5-P, Ru-5-P, and Xu-5-P. From this the TK transfers "active glycolaldehyde," from the Xu-5-P only, to R-5-P, giving sedoheptulose-7-phosphate (SH-7-P) and a molecule of glyceraldehyde-3-phosphate (FIGURE 7). This behavior is stereochemically in agreement with that of the other

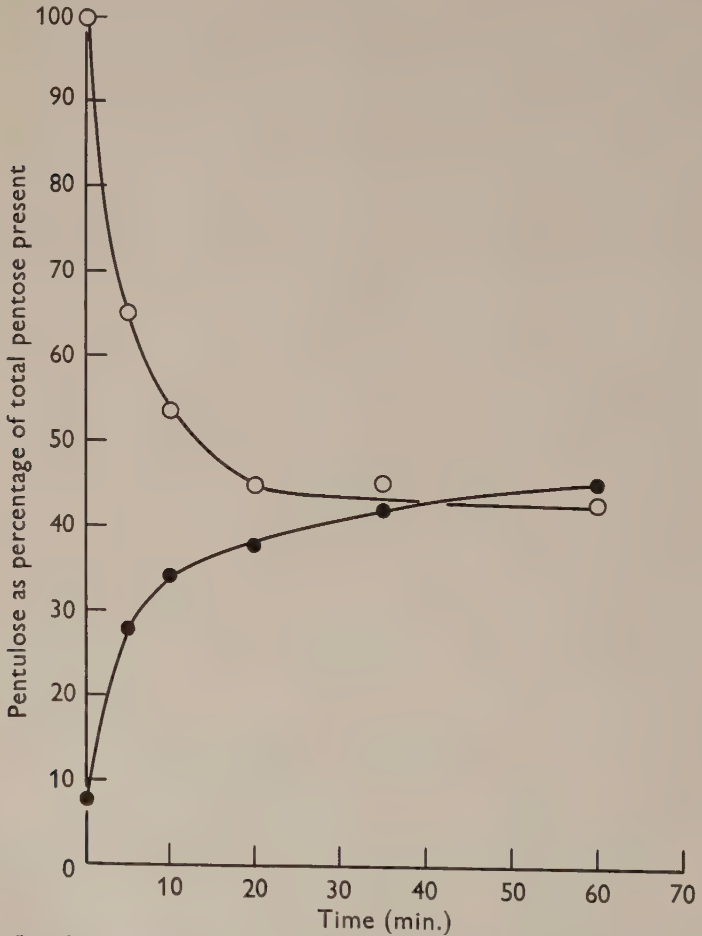
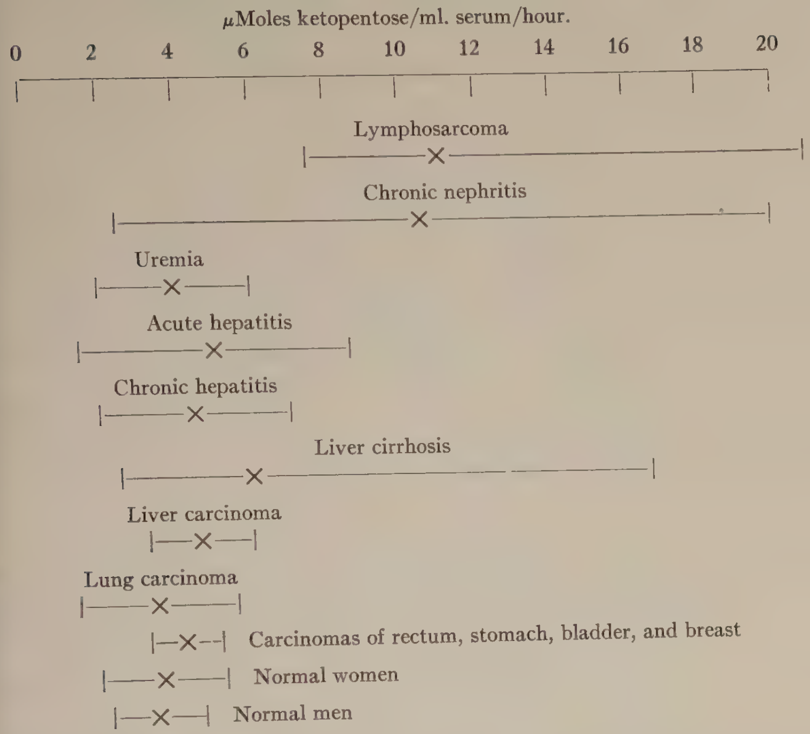


FIGURE 5. The rate of formation of ketopentose phosphate from aldopentose phosphate (R-5-P), and the reverse reaction. In the presence of the mixed pentose phosphate isomerase and epimerase from skeletal muscle: *p*H 7.4, 37° C., and substrate concentrations 1.25 μmole/ml. Line with open cirlces is for a substrate of 90 per cent Ru-5-P and 10 per cent Xu-5-P; line with closed circles is for a substrate of 100 per cent R-5-P. Reproduced from Dickens and Williamson (1956) with the permission of the *Biochemical Journal.*



5-Phosphoribose isomerase in serum (Bruns, 1956)

FIGURE 6. The formation of ketopentose phosphate from ribose-5-phosphate (R-5-P) in human serum (Bruns, 1956). Incubation period, 10 min. at 37° C. with 0.01 molar R-5-P in tris buffer at *p*H 7.5. Colorimetric ketopentose estimations.

known substrates of TK (FIGURE 8). As with aldolase (which, however, also requires the phosphorylation of the C-1 hydroxyl), the occurrence of an OH—group with the L-configuration at C-3 of the D-ketose sugar phosphate is now found to be essential, the exception being hydroxypyruvic acid, which has been shown to be a substrate for transketolase by both Racker's and Horecker's groups (Horecker *et al.*, 1953, Haba *et al.*). This acid, in the presence of carboxylase, yields L-erythrulose (Dickens and Williamson, 1957; Dickens, 1957). These TK reactions require thiamine pyrophosphate and are reversible, except in the case of hydroxypyruvate, where the reversal (CO_2 fixation into L-erythrulose) has not yet been demonstrated.

The acceptor aldehydes for TK, to which the "active" ($CH_2OH \cdot CHO$) group is transferred, include glycolaldehyde, glyceraldehyde, desoxyribose-5-phosphate, glyceraldehyde-3-phosphate (G-3-P), erythrose-4-phosphate, ribose-5-phosphate and, probably, allose-6-phosphate. The products formed by TK action include L-erythrulose (already mentioned), D-xylulose-5-phosphate (with D-glyceraldehyde-3-phosphate), D-fructose-6-phosphate (with D-erythrose-4-phosphate), sedoheptulose-7-phosphate (with ribose-5-phos-

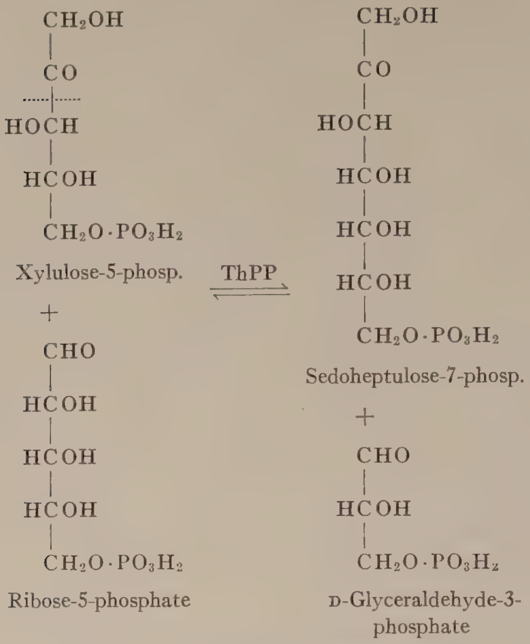


FIGURE 7. The basic transketolase reaction.

phate), and an octulose-8-phosphate (with an undetermined hexose-6-phosphate. Racker and Schroeder, 1957). No doubt this already considerable list will be increased in the future, although (as with aldolase) it does not follow that physiologically significant reactions are always concerned. Of special importance is the proof by Racker *et al.* (1954) that fructose-6-phosphate (F-6-P) is a donor of active glycolaldehyde and that it forms D-erythrose-4-phosphate (REACTION 3). This complement of the reaction, yielding sedoheptulose-7-phosphate from the tetrose phosphate, can proceed by the action of transaldolase [see (TA) as in REACTION 4 below], and as shown originally by Horecker and Smyrniotis:

Fructose-6-phosphate + D-glyceraldehyde-3-phosphate $\xrightleftharpoons{\text{TK}}$
D-xylulose-5-phosphate + D-erythrose-4-phosphate. (3)

D-Erythrose-4-phosphate + fructose-6-phosphate $\xrightleftharpoons{\text{TA}}$
sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate. (4)

Sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate $\xrightleftharpoons{\text{TK}}$
D-xylulose-5-phosphate + D-ribose-5-phosphate (5)

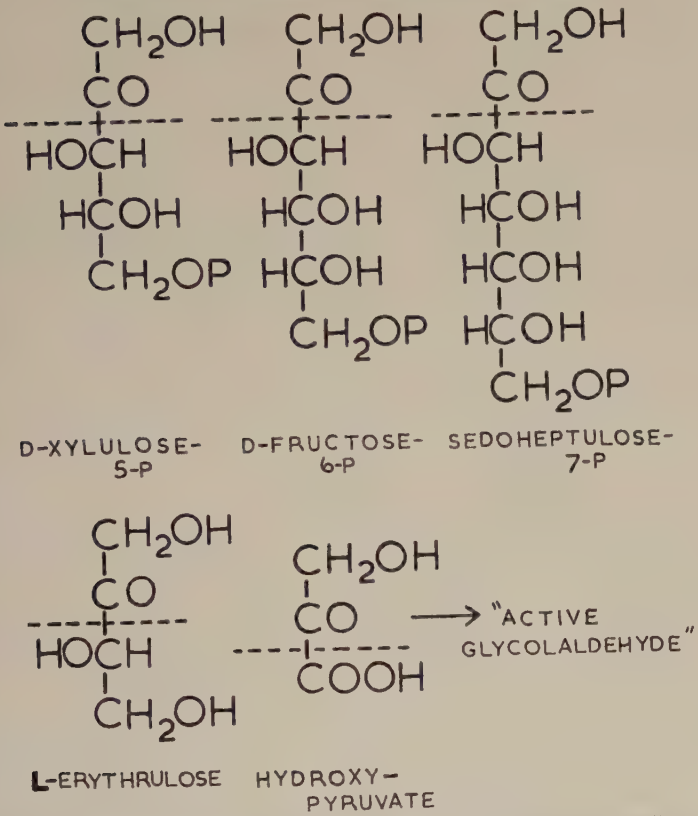


FIGURE 8. The stereospecificity of transketolase. The substrates are split as indicated by the broken lines. D-Ribulose-5-phosphate, which has *cis*-OH groups, is not split, whereas D-xylulose-5-phosphate is a substrate and, adjacent to the carbonyl groups shown above, has a stereochemical configuration similar to that of other substrates of transketolase.

Sum:

$$\text{2-Fructose-6-phosphate} + \text{D-glyceraldehyde-3-phosphate} \rightleftarrows 2\ \text{D-xylulose-5-phosphate} + \text{D-ribose-5-phosphate}. \qquad (6)$$

In this way the interconversion of hexose and pentose phosphate appears to be a remarkably economic and freely occurring reversible process (FIGURE 9). Evidence in support of the physiological importance of reactions of this type will now be presented.

The equilibria constants for transketolase and transaldolase cannot be very confidently stated in view of the uncertainties about the exact substrates involved and the very marked effect on the equilibria of contaminating enzymes (aldolase, PPI, and PKPE). With purified yeast transaldolase, Horecker and Smyrniotis calculate an equilibrium constant at 25° C. of 0.8 for the reaction

$$K = (\text{F-6-P})\cdot(\text{tetrose P})/(\text{SH-7-P})\cdot(\text{glyceraldehyde-3-P}) \qquad (7)$$

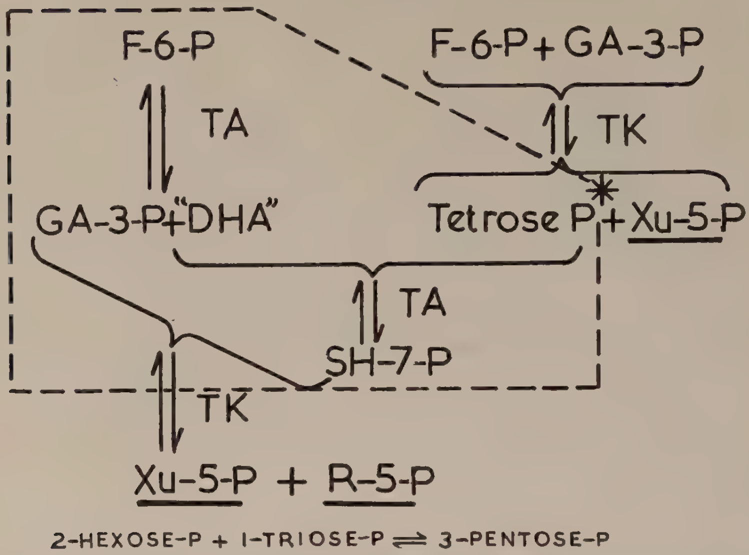


FIGURE 9. Alternative formation of pentose phosphate from hexose phosphate by transketolase and transaldolase. The broken line includes the transaldolase portion of the over all reaction. As in FIGURE 3, "DHA" indicates "active dihydroxyacetone" transferred to the tetrose phosphate by TA. Whether the route to SH-7-P via aldolase (marked by asterisk) actually occurs is hypothetical at present as far as animal tissues are concerned.

* Can also condense (through aldolase) with dihydroxyacetone phosphate (DHAP) → SH-1:7-DP → SH-7-P (phosphatase).

while, with purified spinach transketolase, Horecker *et al.* (1956) calculate that

$$K = (\text{F-6-P})\cdot(\text{GA-3-P})/(\text{Xu-5-P})\cdot(\text{erythrose-4-P}) = \text{approx. unity} \quad (8)$$

It is evident that these reactions are quite readily reversible, and both enzymes can therefore function efficiently in the above scheme.

Various changes can be rung on this theme; thus D-erythrose-4-phosphate (now obtained synthetically by Ballou *et al.*) and dihydroxyacetone phosphate are rapidly and reversibly condensed by aldolase to give D-sedoheptulose-1,7-diphosphate (Horecker *et al.*, 1955) which, if a suitable phosphatase proves to be present, could give an alternative route to SH-7-P.

However, it now appears likely from careful studies *in vivo* of isotopic carbon distribution in which the C^{14} distribution in glucose of glycogen and in ribose of ribonucleic acid (RNA) was compared that in liver, at any rate, the main route to ribose phosphate is by TK and TA reactions similar to those given in REACTIONS 3, 4, and 5 above, with a decarboxylative oxidation at C-1 of glucose as a secondary mechanism of pentose phosphate formation.

From careful experiments on the intact chick fed specifically C^{14}-labeled acetate, glycine or formate, Bernstein concluded that the oxidative HMP route resulting in loss of C-1 of the glucose could not account for the C^{14} distribution found on degradation of the ribose of visceral RNA, since it had no such obvious relationship to the C^{14} distribution actually found on degradation

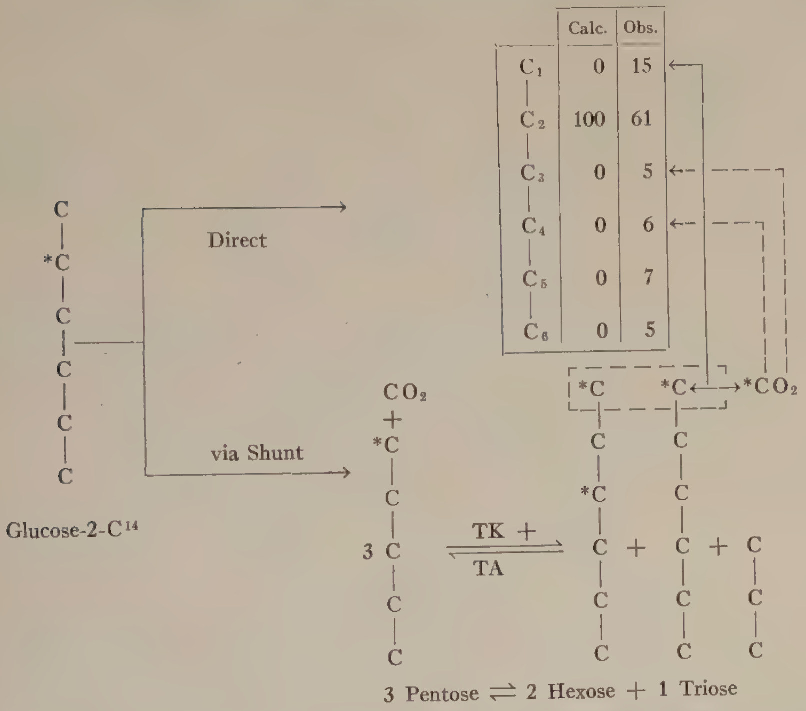


FIGURE 10. The incorporation of glucose-2-C^{14} into the glycogen of rat liver (after Marks and Feigelson, 1957). The column headed "Calc." shows the per cent distribution of C^{14} in the carbon atoms of glucose molecules of liver glycogen as expected for direct incorporation. The observed distribution and the suggested scheme of incorporation are indicated diagrammatically.

of the glucose of visceral glycogen. However, Horecker and Mehler brilliantly analyzed these results on the basis of the TK-TA sequence described above and showed that, in fact, an excellent over-all agreement existed with the C^{14} distribution to be expected on this basis.

Similar and rather convincing results have been reported by Marks and Feigelson, who studied the incorporation of glucose 2-C^{14} into rat liver glycogen (FIGURE 10) with clear indications of alternative pathways in addition to direct incorporation. They also studied the labeling of the isolated ribose of liver RNA (FIGURE 11). In both cases the results were in general accord with a predominantly TK-TA pathway, accompanied by a rather less active HMP C-1 oxidative decarboxylative pathway, as the source of pentose phosphate. These authors explain the unexpectedly low C^{14} incorporation at 3-C of glucose on the basis of a greater rate and extent of TK reactions compared with TA REACTION 4 above.

The converse of this, namely, the conversion, in the mouse, of pentose to glucose of liver glycogen, has been carefully studied by Hiatt. D-Ribose-1-C^{14} was readily incorporated into liver glycogen (FIGURE 12). A specific phosphokinase for D-ribose (FIGURE 12) has been purified from calf liver by Agranoff

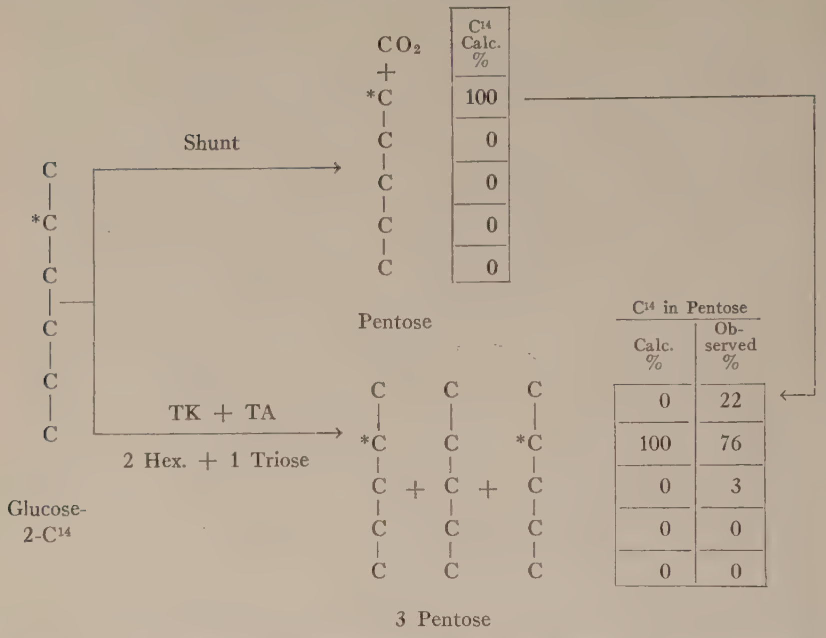


FIGURE 11. The incorporation of glucose-2-C^{14} into the ribose of liver ribonucleic acid (after Marks and Feigelson, 1957). In this case the term "shunt" indicates the oxidative HMP pathway, while the TK + TA pathway represents the nonoxidative conversion of hexose to pentose, as shown in FIGURE 9.

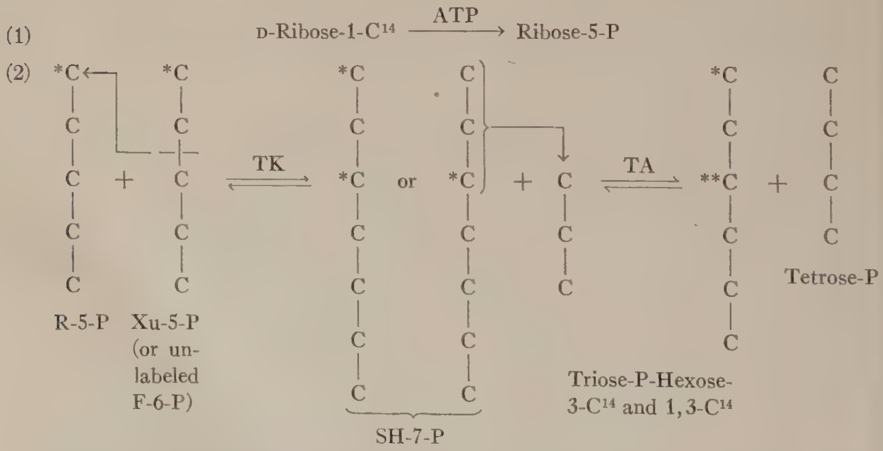

(1)
D-Ribose-1-C14
ATP
Ribose-5-P
(2)
TK
or
TA
R-5-P
Xu-5-P (or unlabeled F-6-P)
SH-7-P
Triose-P-Hexose-3-C14 and 1,3-C14
Tetrose-P

FIGURE 12. The incorporation of ribose-1-C^{14} into the glycogen of rat liver. (1) The action of ribokinase. (2) The course of TK-TA reactions, with expected distribution of C^{14} in the glucose of liver glycogen, as indicated by the asterisks. In the first TK reaction either xylulose-1-C^{14}-5-phosphate (formed from the ribose-5-phosphate by the consecutive action of PPI and PKPE) or unlabeled fructose-6-phosphate (from the pool of hexose phosphates in liver) could partake. The former would yield hexose-1-3-C^{14}, as shown (see FIGURE 2); the latter would give hexose-3-C^{14} (see FIGURE 9). The tetrose phosphate could also undergo a TK or TA reaction.

and Brady. The phosphorylated pentose could be transformed as in FIGURE 12, by the TK-TA system of the liver, the relative extent of labeling in C-3 and C-1 of the resulting glucose depending on the isotope dilution at C-1 introduced by transketolation of unlabeled liver F-6-P participating instead of the C-1-labeled pentose phosphate. FIGURE 13 shows the actual results and the good agreement of C^{14}-distribution in the glucose of liver glycogen with that expected from the TK-TA scheme represented in FIGURE 12.

Evidence from Enzyme and Coenzyme Distribution and the Use of Labeled Substrates

The quantitative distribution in various animal tissues of enzymes of the hexosemonophosphate pathway (G-6-P and 6-PG dehydrogenases and pentose phosphate utilization) was discussed and compared with their glycolysis of glucose ($Q_{CO_2}^{N_2}$) and hexokinase activity in a previous review (Dickens, 1955). In our laboratory Glock and McLean have extended these observations (Glock and McLean, 1955a, b, c; Glock *et al.*, 1956a, b) to include data on the fairly pronounced effects of hormonal balance of the animal on these levels. In animal tumors (FIGURE 14) the usually very active enzymatic utilization of R-5-P is noteworthy, being comparable with that of embryonic tissues, as previously mentioned (Dickens, 1955) in connection with the Walker rat carcinoma. Barron *et al.* (1955) have also drawn attention to this property in intact cancer cells, while suggesting that it may be due to CO_2 fixation into ribulose-1,5-diphosphate. The two dehydrogenases of the HMP pathway are also present in all of the tumors studied, although generally at a lower level of activity (FIGURE 14).

The quite remarkable increase in HMP-cycle enzymes discovered in lactating mammary glands of the rat by Glock and McLean (1953, 1954) has been followed up by studies of glucose-C^{14} metabolism that fully support the great functional activity of the HMP pathway in this tissue, in the respiration of

Glucose-carbons	C^{14} Expected	Observed %	
		A	B
C_1	+(++)*	34	42
C_2	—	4	4
C_3	+++	52	42
C_4	—	4	5
C_5	—	3	3
C_6	—	3	3

FIGURE 13. The incorporation of ribose-1-C^{14} into liver glycogen (Hiatt), showing the observed distribution of C^{14} in the glucose carbons of liver glycogen.

* According to the dilution at C-1 by F-6-P: (A) orally with glucose; (B) subcutaneously with ribose (both nonisotopic).

which glucose-1-C^{14} yields about 10 times as much $C^{14}O_2$ as does glucose-6-C^{14} (Abraham *et al.*, 1954; Glock *et al.*, 1956b; also see FIGURE 15). These results have been extended by Abraham *et al.* (1957), who have made the interesting observations that C^{14} from glucose-3, 4-C^{14} not only yields less $C^{14}O_2$ than glucose-1-C^{14}, but is also incorporated into the fatty acids of lactating mammary glands. This would not be expected to happen via the Embden-Meyerhof and Krebs cycle route; for instance, the C^{14} of carboxyl-labeled lactate is not incorporated in this way. It seems clear that these results, taken together, establish the existence of an active alternative pathway in the lactating gland. The facts indicate that this is the HMP route; at any rate, the glycolytic pathway cannot account for the findings. It is well known that the lactating

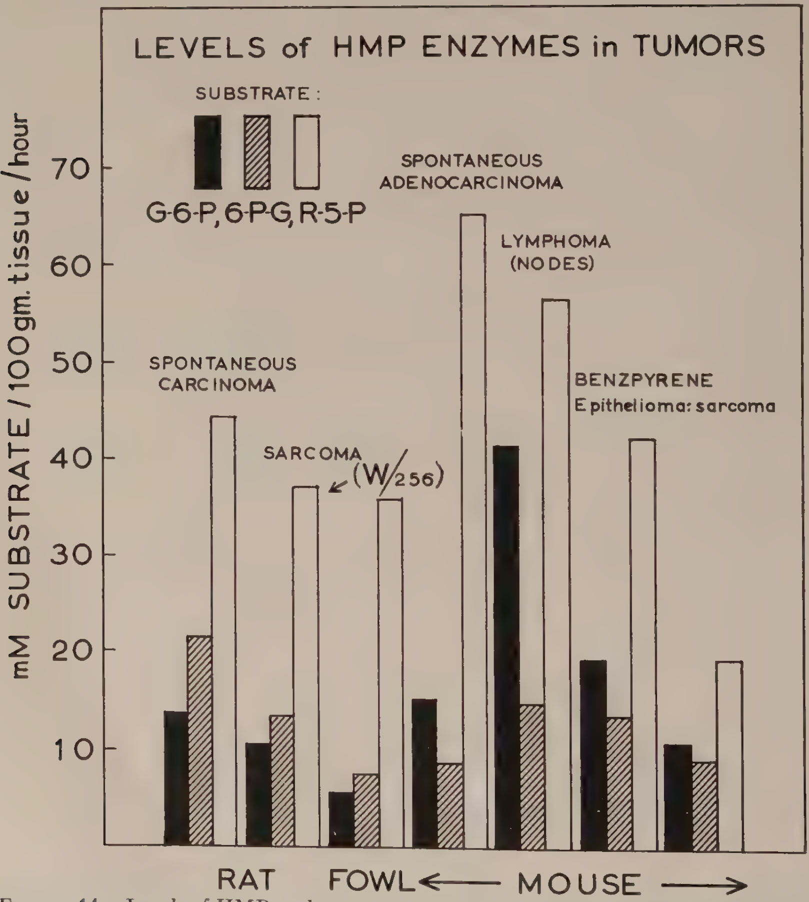


FIGURE 14. Levels of HMP-pathway enzymes in tumor tissues. The data of Glock and McLean (1954) have been calculated for mmoles of substrate converted per hour per 100 gm. (fresh weight) of tissue at 37° C. The Q values (μl./mg. dry weight/hour) can be calculated by multiplying the given values by 1.1, assuming that the dry weight is 0.2 times the wet weight.

gland tissue *in vitro* shows marked effects of added insulin, which appears to increase glucose utilization still more by the alternative pathway than by the glycolytic one (Abraham *et al.*, 1957). After intravenous injection of glucose-1-C^{14} and glucose-6-C^{14} into lactating cows the distribution of isotope into various metabolites, including milk fat, also supports the existence of a pentose phosphate cycle (Black *et al.*).

In corneal epithelium, which has been extensively studied by Kinoshita, a very active HMP system appears to be capable of being coupled through TPN either with glutathione reductase (see Dickens, 1955) or with lactic dehydrogenase, whereby there occurs a dismutation reaction in which the dehydrogenation of G-6-P and 6-PG is coupled with reduction of pyruvate to lactate (Kinoshita). Anaerobically, this tissue yields *no* $C^{14}O_2$ from glucose-6-C^{14} under conditions where considerable $C^{14}O_2$ is liberated from glucose-1-C^{14}. Aerobically a very high ratio of $C^{14}O_2$ production from glucose-1-C^{14}/glucose-6-C^{14} is also characteristic of this tissue (Kinoshita *et al.*).

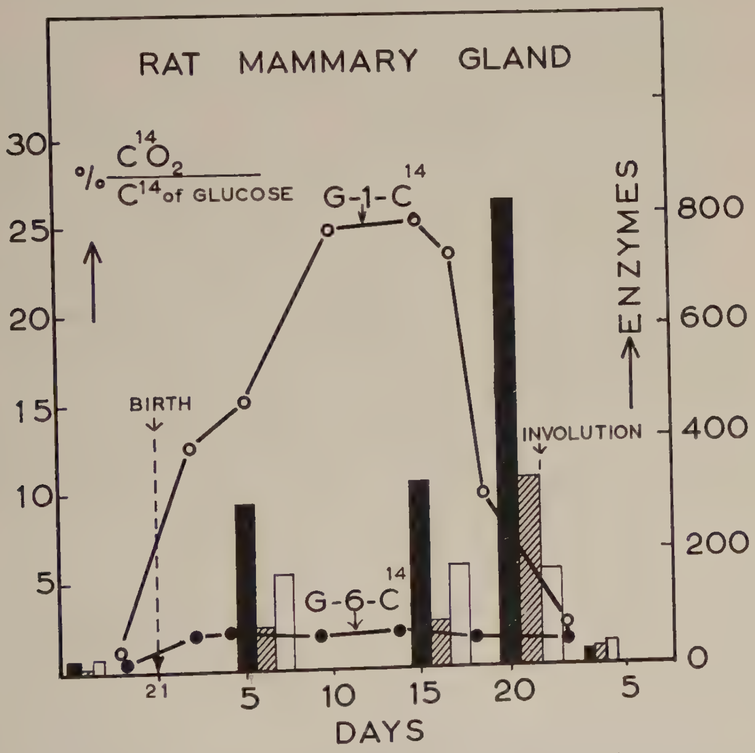


FIGURE 15. The liberation of $C^{14}O_2$ from glucose-1-C^{14} in tissue of the mammary gland of the rat at various periods of lactation (Glock *et al.*, 1956b). The line with the open circles indicates the percentage of liberation of $C^{14}O_2$ from glucose-1-C^{14}; the line with the solid circles indicates the percentage from glucose-6-C^{14}. The histogram, which utilizes the same units and the same enzyme designations as FIGURE 14, illustrates the striking increase in HMP-pathway enzymes during active lactation (Glock and McLean, 1953, 1954).

The method of using the $C^{14}O_2$ liberation from oxidation of specifically labeled substrates, particularly glucose, has been very widely used with a view to determining the respective extents of glycolytic (or Embden-Meyerhof) pathways and HMP (or pentose phosphate) pathways in many types of cells and tissues. Although these experiments are simple to perform, their quantitative interpretation is very difficult and, at present, is perhaps almost a matter of opinion inasmuch as several important assumptions involved in this type of calculation are probably invalid, as has been lucidly pointed out by Wood. At the time of his review, moreover, it was generally assumed that the HMP pathway *necessarily* began with the oxidative loss of C^{14}-1 of the G-6-P as $C^{14}O_2$, followed by the TK-TA reactions on the resulting pentose phosphate. We have already seen that these pentose phosphates can arise equally well by the TK reaction on F-6-P and the subsequent transformations of the pentose phosphate cycle, leading once more to hexose-6-phosphate having quite a different labeling from the original glucose molecule (for example, compare the results of Marks and Feigelson on liver glycogen; also, see FIGURES 10, 11, and 12). This appears to proceed rapidly in tissues such as liver and quickly produces a mixing of the hexose and pentose pools. The over-all result of a combined oxidative and pentose cycle transformation would be that, as pointed out by Korkes, a redistribution of glucose-C^{14} carbon atoms numbers 1 to 6 could occur quickly, and in which the hexose formed might for example, have the type of isotopic pattern shown in TABLE 1. Further mixing could result, for example, from action of transaldolase on F-6-P. This cyle could be repeated with a further redistribution similar to the recycling through the Krebs cycle that is commonly supposed to account for the random distribution of C^{14} in liver glycogen as observed after pyruvate-C^{14} administration. Further transformations, causing isotope to appear in C-4, C-5, and C-6 could also arise through equilibration of the two triose phosphates arising from each hexose molecule by aldolase action.

Finally, if the metabolism of fructose phosphate by way of fructose-1-phosphate aldolase occurs [as in liver, yielding dihydroxyacetone phosphate, (DHAP) and free glyceraldehyde; see Hers and de Duve and Hers] the fate of the free triose will greatly affect the above picture.

TABLE 1

SUGGESTED PATTERN OF DISTRIBUTION OF GLUCOSE-C^{14} CARBON NUMBERS

Original hexose-C atom No.			No. of original hexose-C atom	
(5-Hexose-P)	1	→ (4-Hexose-P)	2212	+ Triose-P + 3 CO_2
	2		3323	
	3		3312	
	4		4444	
	5		5555	
	6		6666	

Apart from the exact nature and equilibration state of the intermediates, another important factor must be the period of the experiment. Beevers and Gibbs showed in 1954 that in yeast cell respiration the ratio C-6/C-1 of yields of $C^{14}O_2$ from glucose-6-C^{14}/glucose-1-C^{14} increased steadily with the time of contact with the respiring cells. However, this factor thus far has received very little consideration. Using slices of a mouse lymphosarcoma and rat heart, van Vals *et al.* recently obtained the following ratios:

	Ratio of $C^{14}O_2$ from G-6-C^{14}/G-1-C^{14} after:			
Tissue	*0.5 hour*	*1 hour*	*2 hours*	*3 hours*
Tumor	0.21	0.35	0.49	0.56
Heart	—	0.93	0.99	1.05

With such marked changes with time as those observed with the tumor, it seems doubtful that elaborate calculations of the percentage metabolism by these two pathways, based on a single experimental period, are of much value.

Consequently, in the following (TABLES 2 and 3) the values of G-6-C^{14}/G-1-C^{14} transformed to $C^{14}O_2$ are to be taken only as a rough qualitative guide to the probable occurrence of an Embden-Meyerhof pathway (C-6/C-1 = 1 approx.) or, in addition, of an oxidative-HMP pathway (ratio below unity). It should be pointed out that values of unity would also be obtained eventually if recycling by the HMP-pentose phosphate pathway occurred, followed by oxidation to CO_2 of all the isotopic glucose carbons.

For the sake of simplicity, experiments with uniformly labeled glucose, or with glucose labeled at other positions (for example, glucose-2-C^{14}; see Agranof *et al.*, 1954), have been omitted from these tables.

Some authors have studied the labeling of aerobically produced lactate from specifically labeled glucose (Wenner and Weinhouse, Villavicencio and Barron). The assumption has been made that C-1 oxidative decarboxylation of the 1-C^{14}-glucose should yield nonisotopic lactate, whereas with 6-C^{14}-glucose the lactate would be labeled by either pathway. However, these assumptions would be invalidated by transketolation of F-6-P as already discussed, by $C^{14}O_2$ fixation, or by the accumulation of pentose phosphate. The results obtained by Wenner and Weinhouse indicated that the pathway of aerobic glucose utilization proceeded principally or almost entirely by the Embden-Meyerhof route, whereas Barron *et al.* conclude that while this was true in normal lymphatic cells from rabbit appendix, in lymphosarcoma cells from the mouse the HMP oxidative pathway plays an important role. An objection to the method based on labeling of lactate is the possibility that oxidative metabolism of glucose (yielding $C^{14}O_2$) might follow one pathway (for example, as an extreme case, an all-HMP route) while aerobic and anaerobic lactic acid production followed the Embden-Meyerhof route. Since, with some tumor

TABLE 2

RATIO OF $C^{14}O_2$ FORMED *IN VITRO* FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} AND LEVELS OF TPN AND TPNH

Normal tissues

Species	Tissue and conditions		Ratio: $\frac{6\text{-}C^{14}}{1\text{-}C^{14}}$	TPNH*	TPN*	Ratio: $\frac{\text{TPNH}}{\text{TPN}}$ (approx.)	$C^{14}O_2$ Reference
Rat	Mammary gland	18-day lactation	0.06	52	<2	(>26)	Glock *et al.*, 1956b
		10 to 14 day lactation	0.14	—	—	—	Abraham *et al.*, 1957
		10- to 14-day lactation + insulin	0.05	—	—	—	Abraham *et al.*, 1957
Rat	Corneal epithelium		0.15	—	—	—	Kinoshita *et al.*
Rat	Adrenal		—	116	17	6	—
Rabbit	Adrenal (whole)		—	62	<2	(>31)	—
Rabbit	Adrenal cortex		—	68	14	5	—
Rat	Testis		0.3	6	<2	(>3)	Bloom
Rat	Bone marrow		0.3	—	—	—	Bloom
Mouse	Spleen		0.35 to 0.57	12	<2	(>6)	Bloom
Rat	Thymus		0.4 to 0.75	9	<3	(>3)	Kit
Mouse	Lung		0.57	—	—	—	Van Vals *et al.*
Rat	Lung		—	18	9	2	—
Rat	Liver	Normal diet	0.33	205	6	35	Agranoff *et al.*
Rat	Liver	High-glucose diet	0.29	—	—	—	Agranoff *et al.*
Rat	Liver	Fasted (24 hr.)	0.36 to 0.78	308	20	15†	Agranoff *et al.*
Rat	Liver	Fasted (48 hr.)		308	<2	>150†	
Rat	Liver	Regenerating	0.54	—	—	—	Agranoff *et al.*
Rat	Liver	Maternal	0.20 to 0.33	—	—	—	Agranoff *et al.*
Rat	Liver	Fetal	0.32 to 0.61	—	—	—	Agranoff *et al.*
Rat	Liver	Normal diet	0.35	—	—	—	Katz *et al.*

Rat	Liver	High K^+ medium	0.68 to 0.87	—	—	—	Ashmore *et al.*
Rat	Liver	Bicarb. Ringer	0.69 to 0.88	—	—	—	Ashmore *et al.*
Rat	Liver	Phosphate Ringer	0.41 to 0.58	—	—	—	Ashmore *et al.*
Rat	Liver	Normal	0.50 to 0.62	—	—	—	Glock *et al.*, 1956a
Rat	Liver	Alloxan diabetic, pair-fed controls	0.27	159 148	8 10	20† 15†	Glock *et al.*, 1956a
Rat	Liver	Pair-fed controls with insulin	0.44	—	—	—	Glock *et al.*, 1956a
Rat	Liver	Thyroxin treated	0.75	155	<2	>75†	Glock *et al.*, 1956a
Rat	Liver	Growth hormone treated	—	196	2	100†	—
Rabbit	Liver		0.28 to 0.40	—	—	—	Bloom
Mouse	Liver		0.9 to 1.0	—	—	—	Abraham *et al.*, 1955
Rat	Kidney		0.6 to 0.9	54	3	18	Bloom
Rat	Kidney		1.01	—	—	—	Van Vals *et al.*
Rat	Heart		0.6 to 0.8	33	4	8	Bloom
Rat	Kidney		1.0	—	—	—	Van Vals *et al.*
Rat	Diaphragm		0.9 to 1.0	13	<2	(>6)	Bloom and Stetton
Rat	Diaphragm		0.98	12	3	4†	Van Vals *et al.*
Rat	Diaphragm (thyroxin treated)		—	15	<2	>7†	—
Rat	Skeletal muscle			9	8	1	—
Mouse	Skeletal muscle			3	<3	?	—
Rat	Brain		0.9 to 1.0	8	<2	>4	Bloom
Rat	Brain		1.08	—	—	—	Van Vals *et al.*
Rat	Blood (whole)		—	5	3	2	—
Rabbit	Blood (whole)		—	3	<2	(>1.5)	—

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* Data of Glock and McLean (1955a) for normal tissues; data of Glock and McLean (1957b) for tumors and skeletal muscles. Coenzymes are expressed as μg./gm. of fresh tissue and not determined on the same samples of tissue as were the $C^{14}O_2$ ratios to which references are cited in the last column.

† Data of Glock and McLean (1955b).

TABLE 3

RATIO OF $C^{14}O_2$ FORMED *IN VITRO* FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14}; AND LEVELS OF TPN AND TPNH

Tumor tissues

Species	Tissue and conditions	Ratio: $\frac{6\text{-}C^{14}}{1\text{-}C^{14}}$	TPNH*	TPN*	Ratio: $\frac{TPNH}{TPN}$ (approx.)	$C^{14}O_2$ References
Rat	Azo-dye liver tumors	0.55 to 0.67	—	—	—	Agranoff *et al.*
Rat	Azo-dye liver tumors	0.38	—	—	—	Van Vals *et al.*
Rat	Azo-dye liver tumors (transplanted)	0.11 to 0.41	—	—	—	Agranoff *et al.*
Rat	Azo-dye liver cholangiomas and hepatomas	—	27	3	9	—
Rat	Azo-dye liver hepatoma	0.33	28	3	>9	Wenner and Weinhouse
[Rat	Azo-dye tumor; adjacent liver tissue		92	4	23]	—
[Rat	Normal liver; average	0.4 to 0.5	217	7	31]	—
Rat	Jensen sarcoma (transplanted)	—	4	<3	(>1)	—
Rat	Walker carcinoma (transplanted)	—	3	<3	?	—
Mouse	Sarcoma 37	0.43	3	<3	?	—
Mouse	Mammary adenocarcinoma	0.27	—	—	—	Wenner and Weinhouse
Mouse	Mammary carcinoma	0.45, 0.50	—	—	—	Van Vals *et al.*
Mouse	Hepatoma	0.32	—	—	—	Wenner and Weinhouse
Mouse	MC/63 Carcinoma	—	3	<3	?	—
Mouse	Ehrlich ascites tumor	0.19	—	—	—	Wenner and Weinhouse
Mouse	Ehrlich ascites tumor	0.22	—	—	—	Kit
Mouse	Krebs II ascites tumor	—	3	<3	?	—
Mouse	Leukemia	—	3	<3	?	—
Mouse	Lymphosarcoma	0.21 to 0.56	3	<3	?	Van Vals *et al.*
		0.19 to 0.37	—	—	—	Kit
Mouse	Rhabdomyosarcoma	1.0	—	—	—	Wenner and Weinhouse
Human	Fibrosarcoma	0.14 to 0.4	—	—	—	Van Vals *et al.*

* Data (in μg. coenzyme/gm. fresh tissue) of Glock and McLean (1957b) for tumor coenzyme levels throughout. Reference numbers refer only to ratio of $C^{14}O_2$ on similar material. A query mark under "ratio TPNH/TPN" indicates that coenzyme levels are too low to permit calculation of a ratio.

tissues, the aerobic lactate production uses about 20 times as much glucose as does the respiration, the maximum possible alteration of the 6-C^{14}/1-C^{14} oxidation ratio would presumably have only a trifling effect on the labeling of the lactate produced. The method of expressing the results as a percentage of the *total glucose* used by the cell going through the Embden-Meyerhof route would therefore not necessarily give a true picture of the *respiration* in a tissue having high aerobic glycolysis. Finally, of course, the possibility of as yet undiscovered metabolic routes in any particular tissue must always be kept in mind.

With these reservations we may now briefly consider the values of glucose-6-C^{14}/glucose-1-C^{14} given in TABLE 2 for normal tissues and TABLE 3 for tumor tissues. The lowest ratios recorded are for lactating mammary gland and corneal epithelium, in which independent evidence, already summarized, strongly supports a vigorous HMP pathway. A group of tissues with a 6-C/1-C ratio of 0.3 to 0.6 follows (TABLE 2). Liver, in which tissue the effects of diet and other experimental conditions have been much explored, shows very variable ratios of 0.2 to 0.8. According to most of the observations, kidney, heart, diaphragm, skeletal muscle, and brain all give ratios approaching unity. Finally, all the tumors reported in TABLE 3, with the sole exception of one rhabdomyosarcoma, have yielded 6-C/1-C ratios less than unity, frequently in the region 0.1 to 0.4.

It is instructive to compare these ratios with the levels of coenzymes, particularly of oxidized and reduced TPN, as determined by Glock and McLean (1955a, b, c; 1957b). Although many gaps remain to be filled, data included in TABLES 2 and 3 illustrate the following points: (1) Several normal tissues with a low ratio of glucose-6-C/glucose-1-C liberation as CO_2, particularly lactating mammary gland and liver, also have a high content of total TPN. Brain, skeletal muscle, and diaphragm have very low amounts of total TPN, and these tissues have generally been reported as having 6-C/1-C quotients near unity. (2) The results of Glock and McLean (1955a, b, c) show that, whereas the DPN is present mainly in the oxidized form, the TPNH greatly exceeds the oxidized TPN in all these tissues. (3) In the tumors studied (TABLE 3 and FIGURE 16), the total amount of TPN is very low, being often too small to estimate. The total DPN content of tumors (Glock and McLean, 1957b), on the other hand, falls about in the middle of the range reported for normal tissues and is remarkably constant (range 133 to 174 μg./gm. tissue). Nevertheless, with few exceptions, tumors yield low 6-C/1-C ratios (TABLE 3), suggesting an active HMP-pentose phosphate pathway. The dehydrogenases in tumor tissue for G-6-P and 6-PG have been found in our laboratory to be TPN-specific, like those in normal animal tissues, and the pyridine nucleotide transhydrogenase activity of tumors (which might enable a low TPN content to act through the DPN present) and TPNH-cytochrome *c* reductase activity are both also quite weak in liver tumors (Glock and McLean, personal communication).

If there is an active pentose phosphate pathway in tumor tissue it is therefore difficult to picture it as proceeding by the oxidative decarboxylation of HMP, for which an adequate level of TPN, and probably of oxidizing systems acting on TPNH, seem to be lacking. Barron *et al.* (1955), in a brief communication, found that tumor cells could utilize ribose-5-phosphate rapidly under anaerobic conditions, and that this was accompanied by $C^{14}O_2$-fixation into the carboxyl of lactic acid. They believed that this occurred by a mechanism similar to photosynthetic fixation; that is, via ribulose-1,5-diphosphate, and carboxydismutase. If this is indeed the mechanism, it would be a very important finding, but the available evidence is insufficient until other more familiar routes of CO_2 fixation have been excluded. The most obvious available source for ribose-5-phosphate formation from hexose in tumors would appear to be by way of a transketolase-transaldolase sequence similar to that shown in FIGURE 9. However, the very low 6-C/1-C ratios in tumor tissue still require explana-

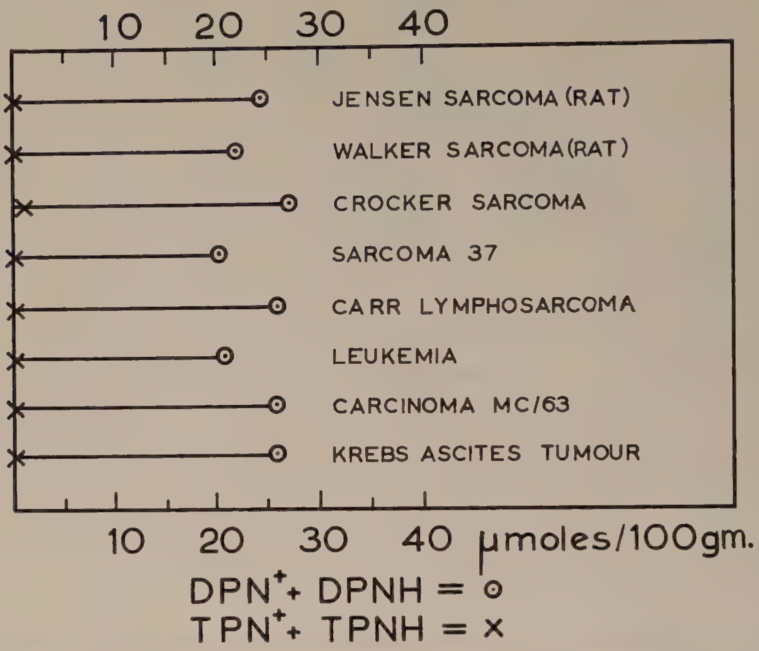

FIGURE 16. The levels of coenzymes in animal tumors. This chart shows the total levels oxidized and reduced, of DPN and TPN, as calculated from the data of Glock and McLean (1957b). The extremely low levels of TPN that are shown may be compared, for example, with those of brain tissue or of diaphragm or skeletal muscle; however, they are much lower than those found by the same authors (1955a) in the liver, the adrenal, the kidney, the ovary, and the lactating mammary gland.

tion. It seems that the Entner-Doudoroff type of cleavage of hexose is excluded, because Villavicencio and Barron (1956) found that, in lymphatic cells and lymphosarcoma cells, glucose-1-C^{14} gave lactate with less than 1 per cent of the total C^{14} in the COOH group, instead of the 100 per cent that would be expected by this pathway. The problem of how the tumor tissue can combine its low 6-C/1-C ratio with its extremely low content of TPN therefore remains unsolved at present: this may prove a weak spot in tumor metabolism.

Hers (1955) has found that $C^{14}O_2$ liberation from glucose-1-C^{14} by liver slices is much increased (compared with that from G-U-C^{14}) on addition of substrates of the TPN-linked aldose reductase present in liver, such as glyceraldehyde and glucosone. The theory put forward (de Duve and Hers, 1957) to explain this is that the HMP oxidative pathway is limited by the amount of TPN which is present in the oxidized form. An increased glucose-1-C^{14} oxidation should therefore result from a fall in the level of the ratio TPNH/TPN^+. TABLE 2 shows some values of this ratio for those few tissues for which they are at present available. The value can not be calculated where the data show very low TPN values, particularly in the case of most tumors (TABLE 3), and even in the other instances is only approximate. However, the calculated ratios (TABLE 2) appear to be too variable for any definite correlation of this kind, although this type of control mechanism cannot be excluded, particularly since it has been found by Glock and McLean (1957a) that the ratios of TPNH/TPN$^+$, as in rat liver homogenate, for example, may vary from about 9:1 in the whole homogenate to 30:1 in the mitochondria, and only 3:1 in the soluble fraction. In this connection, data on the comparative activity of the TPNH-cytochrome *c* reductase in various tissues would be very valuable.

In conclusion, an attempt to detect transketolase pathways in intact cells and in the whole animal has been made by studying the fate of 3-C^{14} and 2-C^{14}-labeled hydroxypyruvate (Dickens, 1957; Dickens and Williamson, 1958). This substance, as already described, is a good substrate for transketolase in isolated systems, but *in vivo* we find that it is deposited in liver glycogen in a manner suggesting strongly that it is mainly incorporated by a different and rather direct pathway, presumably through 3-phosphoglycerate. Degradation of glycogen-glucose showed labeling, located mainly at 1-C, 2-C, 5-C, and 6-C of the glucose molecule, which was in agreement with this type of route, being almost identical with the pattern of incorporation of serine carbon into glycogen as described by Friedman *et al.* (1956). Some pentose formation could be demonstrated in liver slices, but the principal route again appeared to be incorporation into glycose. We find that phosphokinases active towards glyceric acid, glycerol, glyceraldehyde, and dihydroxyacetone do not phosphorylate free hydroxypyruvate, and it may therefore need to be reduced to D-glycerate before phosphorylation and subsequent incorporation; this possibility is now being investigated.*

Acknowledgment

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* A further mechanism may be based on the finding that formaldehyde acts as an "acceptor" for transketolase. (Dickens and Williamson, 1958b).

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STUDIES ON THE MECHANISM OF AGING OF HUMAN RED BLOOD CELLS*

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The maintenance of the structural integrity of nonnucleated erythrocytes appears to be dependent on energy-yielding processes.[1] Shemin and Rittenberg[2] demonstrated that under normal circumstances the destruction of human red blood cells is related to their senescence. These observations suggest the possibility that the aging of erythrocytes and their eventual disintegration may result from a progressive diminution in the activity of certain essential reactions. The studies reviewed here were designed first, to determine whether alterations in the levels of enzymes occur in association with the *in vivo* aging of red blood cells and, second, to measure various metabolic processes, such as glucose oxidation and lipid synthesis, in young erythrocytes as compared with old ones.

Several previous investigators, including Sabin,[3] Pritchard,[4] and Rubinstein *et al.*,[5] have studied the changes that occur in enzyme activity in the reticulocyte during its maturation into the erythrocyte. These workers have found that the levels of activity of certain, but not all, red blood cell enzymes are elevated in cell populations containing a high percentage of reticulocytes. In addition, Allison and Burn[6] provided evidence suggesting that cholinesterase and catalase activities fall progressively in transfused donor cells as they age *in vivo*. Recent observations by Jaffé *et al.*[7] suggest that nucleoside utilization is greater in young than old erythrocytes.

In the present studies we first directed attention toward the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. These enzymes catalyze the reactions:†

$$\text{glucose-6-P} + \text{TPN} \xrightleftharpoons[\text{dehydrogenase}]{\text{glucose-6-P}} \text{6-phosphogluconolactone} + \text{TPNH} + \text{H}^+ \qquad (1)$$

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† The following abbreviations are employed: TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

$$\text{6-phosphogluconic acid} + \text{TPN} \xrightleftharpoons[\text{dehydrogenase}]{\text{6-phosphogluconic}} \text{ribulose-5-P} + \text{TPNH} + \text{H}^+ + \text{CO}_2 \qquad (2)$$

The mature erythrocyte lacks many of the enzymes of the tricarboxylic acid cycle. Accordingly, these dehydrogenases provide the only important mechanism in erythrocytes for glucose oxidation to CO_2 with generation of reduced triphosphopyridine nucleotide.[8] As indicated in REACTIONS 1 and 2, for every mole of hexose phosphate oxidized, a mole of TPNH is formed. This fact is the basis of the assay for these enzymes. The rate of TPNH formation is determined in a spectrophotometer at 340 mμ.[9, 10]

There have been no previous reports of the activity of erythrocyte glucose-6-phosphate and 6-phosphogluconic dehydrogenases in human erythrocytes in relation to maturation or aging of these cells. Rubinstein *et al.*[5] determined erythrocyte glucose-6-P dehydrogenase in rabbits with reticulocytosis induced by phenylhydrazine administration and concluded that this enzyme was about equally active in the reticulocyte and the mature erythrocyte.

Erythrocyte Enzymes in Patients with Reticulocytosis

In patients with reticulocytosis, the levels of both erythrocyte glucose-6-phosphate and 6-phosphogluconic dehydrogenases were found to be significantly elevated (FIGURE 1).[11, 12] In the group of 112 normal subjects indicated in FIGURE 1 the level of glucose-6-phosphate dehydrogenase was 15.9 ± 2.4 units* compared with a value of 29.7 ± 2.4 units for the patients with reticulocytosis. The level of 6-phosphogluconic dehydrogenase in 66 normal persons was 17.3 ± 3.90 units compared with 36.2 ± 8.70 units for the patients with reticulocytosis. The patients with reticulocytosis studied had a wide variety of conditions, including acquired and congenital hemolytic anemias, blood loss, pernicious anemia, sprue, and neoplastic diseases. In view of these findings of elevated dehydrogenase activities in patients with anemia, the report[13] that erythrocyte purine nucleoside phosphorylase activity was not elevated in three patients with reticulocytosis was of particular interest. Purine nucleoside phosphorylase catalyzes the reaction:

$$\text{purine nucleoside} + \text{inorganic phosphate} \xrightleftharpoons[\text{phosphorylase}]{\text{purine nucleoside}} \text{ribose-1-P} + \text{purine.} \qquad (3)$$

The earlier observation was confirmed and extended with the finding[12] that there was no significant difference between the mean value of erythrocyte pu-

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* These figures are mean values ±1 standard variation. For glucose-6-phosphate dehydrogenase, 6-phosphoglucuronic dehydrogenase, purine nucleoside phosphorylase, and lactic acid dehydrogenase, a unit of enzyme activity is defined as the change in optical density per minute per gram of hemoglobin. For phosphohexose isomerase, a unit of enzyme activity is defined as the reciprocal of the amount of the sample (grams of hemoglobin per milliliter of reaction mixture) which, under these conditions, would produce 25 μg. fructose as fructose-6-phosphate from glucose-6-phosphate.

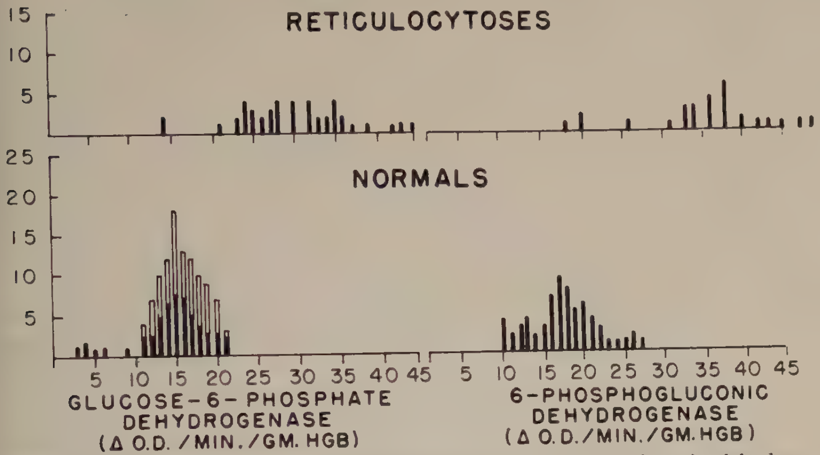

FIGURE 1. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase levels in erythrocytes of normal subjects and patients with reticulocytosis. The data with respect to glucose-6-phosphate dehydrogenase are represented by white bars for Caucasians and black bars for Negroes.

rine nucleoside phosphorylase of 52 normal subjects compared with that of 28 patients with reticulocytosis.

Among normal persons, a certain number had significantly low levels of erythrocyte glucose-6-phosphate dehydrogenase (FIGURE 1). This erythrocyte enzyme deficiency appears to be a genetically determined trait. The implications of this finding are discussed elsewhere in this monograph by Gross and Marks. In the group of normal individuals no significant differences were found between the mean values for glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, or purine nucleoside phosphorylase in erythrocytes of males as compared with those of females, of Caucasians as compared with those of Negroes, or when age groups were compared by decades from 20 to 70 years.

The observation of increased erythrocyte glucose-6-phosphate and 6-phosphogluconic dehydrogenases in association with reticulocytosis raises the question whether the degree of elevation of these enzymes is correlated with the reticulocyte count. However, no significant correlation was found between the degree of reticulocytosis and the level of these dehydrogenases, possibly reflecting the fact that young but nonreticulated cells may have as high activity of these enzymes as do reticulated cells.

The lack of correlation between the degree of elevation in the levels of the dehydrogenases and the percentage content of reticulocytes was substantiated in serial studies of 6 patients with macrocytic anemia prior to and following vitamin B_{12} therapy. Five of these patients had pernicious anemia; 1 had sprue. The data obtained in these cases are illustrated by 2 examples in FIGURE 2. A sharp rise was observed in the level of activity of both glucose-6-phosphate and 6-phosphogluconic dehydrogenases, but not in purine nucleoside phosphorylase, coincident with the reticulocytosis induced by vitamin B_{12} therapy. Although the reticulocyte count returned to normal levels by

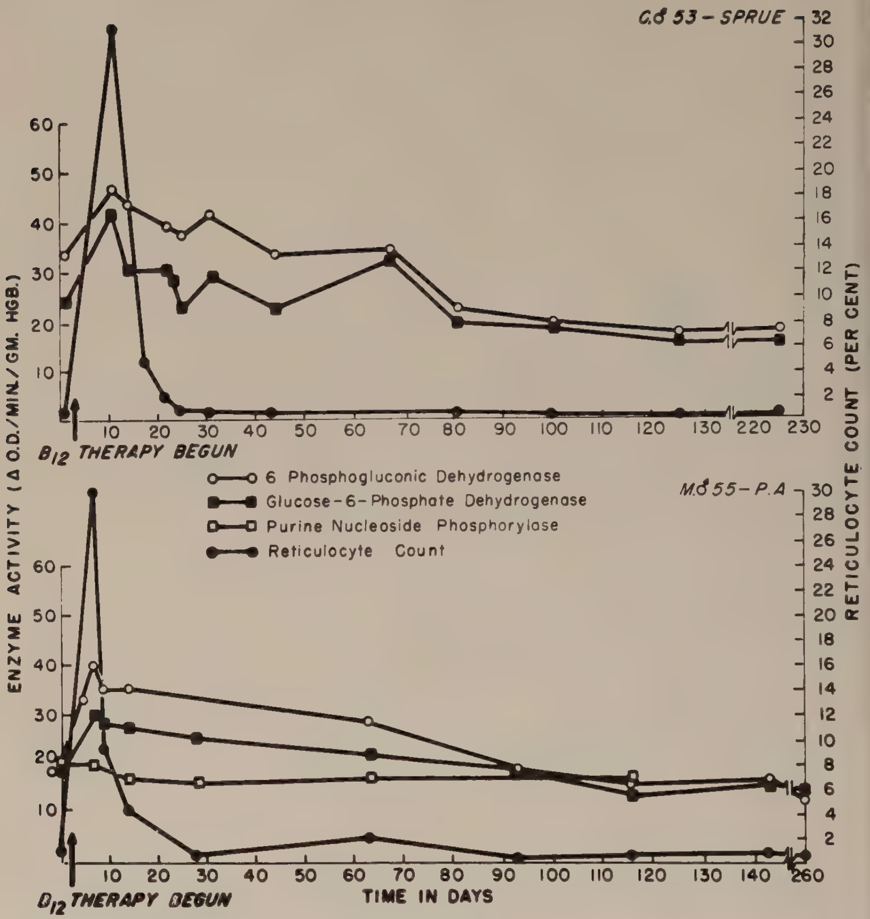

FIGURE 2. Erythrocyte enzyme levels in two patients with macrocytic anemia before and after vitamin B_{12} therapy.

days 20 to 25, the levels of the dehydrogenases remained elevated for 58 to 90 days following the institution of therapy. These findings indicate that high levels of glucose-6-phosphate and 6-phosphogluconic dehydrogenases are sensitive indices of erythrocyte populations with a younger than normal mean cell age. In addition, these data suggest that the activities of glucose-6-phosphate and 6-phosphogluconic dehydrogenases are high in young red blood cells and decrease with the aging of these cells *in vivo.*

Fractionation of Erythrocytes into Young and Old Cells

To determine whether changes in erythrocyte enzyme activity occur as these cells age *in vivo*, techniques have been employed that permit the separation of a sample of erythrocytes into fractions with relatively young and old mean cell ages. These techniques of fractionation are based on certain differences in the characteristics of young red cells relative to old ones. Several observers have noted that the osmotic fragility of young cells is less than that of older cells[14] and also that the density is lower in the younger cells.[15-18] Furthermore, it is established that injected iron-59 will label only newly formed erythrocytes.[19, 20]

In the present studies a technique of serial osmotic hemolysis has been employed to fractionate cells according to age.[21] This technique was validated by determining the concentration of erythrocytes of varying ages as dated by iron-59 labeling *in vivo* in fractions of cells of different osmotic fragility.[22]

The fractionation of the whole red blood cell population by osmotic hemolysis was carried out briefly as follows:[21] at 0° C. washed red blood cells largely freed of plasma, buffy coat, and reticulocytes were added to solutions of varying sodium chloride concentration so as to give graded hemolysis between 2 and 100 per cent. Results obtained in fractionation of samples of erythrocytes from nine subjects are summarized in FIGURE 3 for 2 levels of hemolysis: the 5 per cent most resistant and the 5 per cent least resistant fractions. The mean values for the percentage of erythrocytes of a given age in these fractions are plotted against the age of erythrocytes dated by days following the adminis-

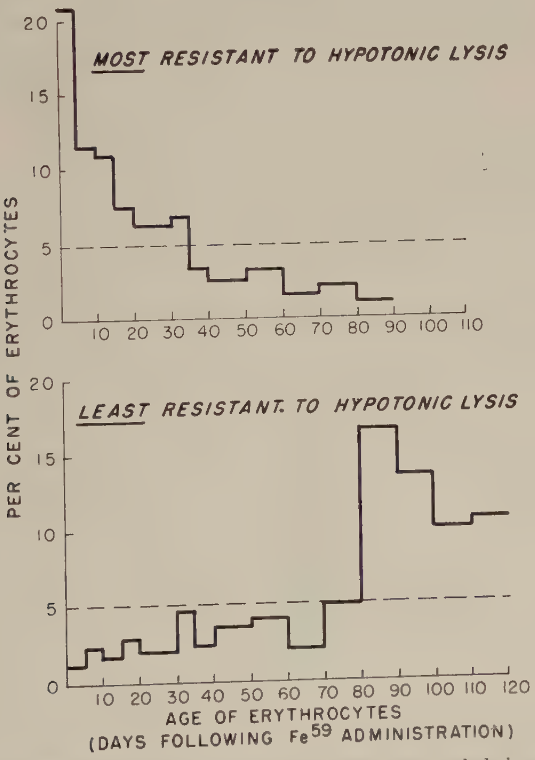

FIGURE 3. Correlation between age of human erythrocytes and their resistance to hypotonic lysis.

tration of iron-59. In this figure, values in excess of 5 per cent indicate enrichment, and values below 5 per cent show impoverishment of the fraction with respect to cells of a given age. The 5 per cent most-resistant fraction thus was enriched with cells 35 days old or younger, and impoverished with regard to cells older than 35 days. In this 5 per cent most-resistant fraction, the younger the cells, the greater the degree of enrichment. On the other hand, the 5 per cent least-resistant fraction was enriched with cells older than 80 days and impoverished with regard to cells younger than 70 days. These data indicate that young mature human erythrocytes are more resistant to hypotonic hemolysis than are old cells. Thus, serial osmotic hemolysis provides a means of separating red cells into fractions of differing mean cell ages.

Erythrocyte Enzyme Activity in Fractions of Differing Mean Cell Age

FIGURES 4 through 8 summarize the data on enzyme activity in relation to the age of erythrocytes.*

The average glucose-6-phosphate dehydrogenase activity in the 2 per cent most-resistant cells, the youngest cell fraction indicated in FIGURE 4, was approximately twentyfold that in the 2 per cent least-resistant cells of greatest mean cell age. The 6-phosphogluconic dehydrogenase activity (FIGURE 5) was 4 times higher in the more resistant younger cell fractions than in the less resistant older fractions. Purine nucleoside phosphorylase (FIGURE 6) was found to be only slightly lower in the less resistant older cells than in the more resistant younger cell fractions.

In view of the fact that erythrocytes derive most of their energy from glycolysis, we also investigated two enzymes of the Embden-Meyerhof pathway. Phosphohexose isomerase, which catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate, and lactic acid dehydrogenase, which catalyzes the reduction of pyruvic acid by reduced diphosphopyridine nucleotide to lactic acid, have both been investigated.

Phosphohexose isomerase (FIGURE 7) activity was about three times greater in the younger, more resistant cells than in the older, less resistant ones. On the other hand lactic acid dehydrogenase (FIGURE 8) was reduced only about 10 to 15 per cent with aging.

These data, obtained with the osmotic hemolysis method of separation, were substantiated by centrifugal fractionation. Centrifugation, in accord with the report of Borun *et al.*,[18] accomplished a partial fractionation of the whole red blood population, though not as effectively as that achieved by osmotic hemolysis.[22] Nevertheless, data obtained with this second technique, as indicated in FIGURE 9, again demonstrated that relatively young compared to old erythrocyte fractions have higher levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase, but not of purine nucleoside phosphorylase.

* Glucose-6-phosphate dehydrogenase was assayed by a method based on that of Kornberg and Horecker,[10] 6-phosphogluconic dehydrogenase by a technique similar to that of Horecker and Smyrniotis,[9] and purine nucleoside phosphorylase by a modification of the method of Price *et al.*[23] The details of these enzyme assays have been described elsewhere.[11] Phosphohexose isomerase was determined according to the method of Bodansky,[24] and lactic acid hydrogenase by a procedure based on the technique of Hill and Levi.[25]

These data indicate that the activity of certain erythrocyte enzymes decreases markedly as these cells age *in vivo*. It should be emphasized that the differences between young and old red cells with regard to the levels of enzymes are very much greater than the differences indicated for the fractions of cells of varying osmotic resistance. These fractions are merely enriched with young or old cells.

The explanation of the decline in enzyme activity with erythrocyte aging *in vivo* is not apparent from our present knowledge. A possible interpretation of these observations is that, following loss of nucleic acids, the erythrocyte cannot synthesize proteins.[26] The previously synthesized complement of enzymes then falls progressively as the cell becomes older. If erythrocytes do indeed synthesize enzymes, then the diminution in activity of a given enzyme with age may be related to alteration in rates of turnover of these proteins. These problems are under investigation.

Oxygen Consumption and Lipid Synthesis in Young and Old Red Cell Fractions

These observations raise a question as to whether alterations in metabolic functions of the intact erythrocyte occur in association with the enzymatic

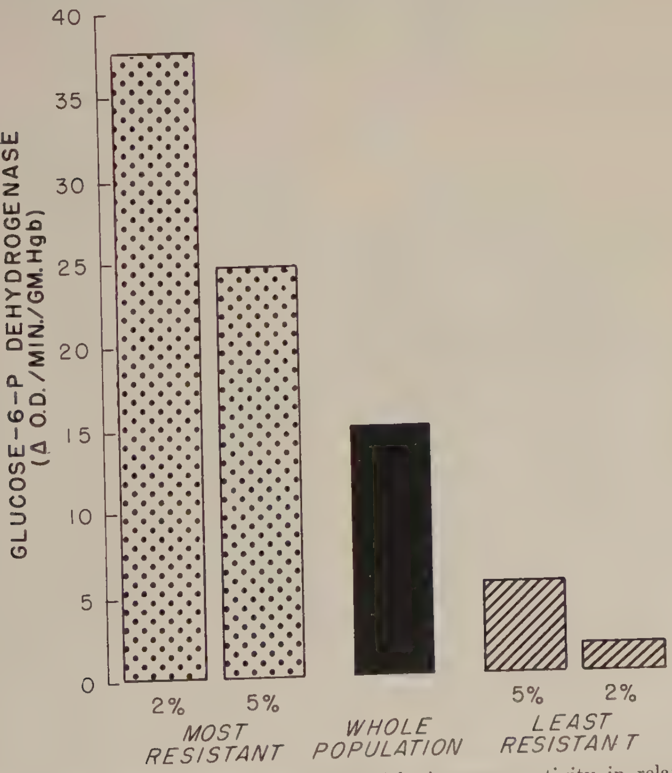

FIGURE 4. Erythrocyte glucose-6-phosphate dehydrogenase activity in relation to osmotic fragility of cells.

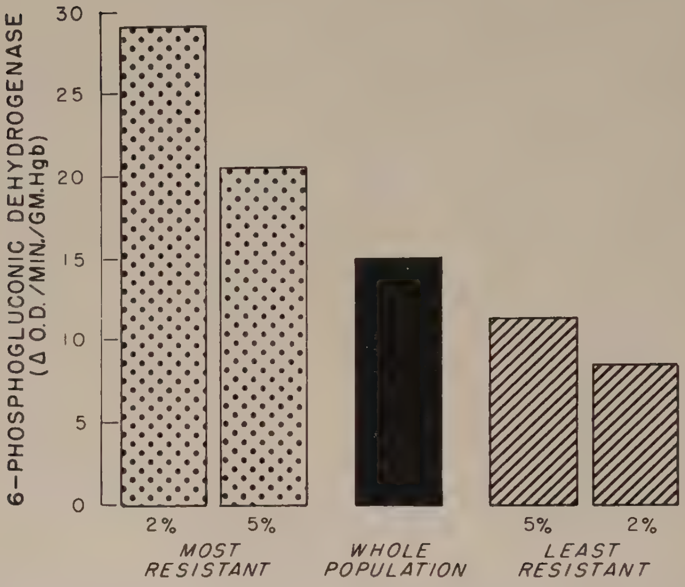

FIGURE 5. Erythrocyte 6-phosphogluconic dehydrogenase activity in relation to osmotic fragility of cells.

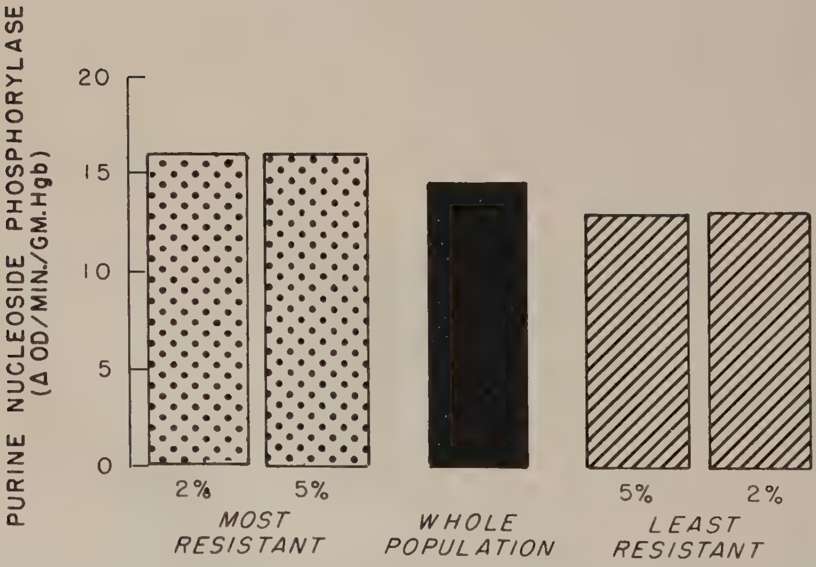

FIGURE 6. Erythrocyte purine nucleoside phosphorylase activity in relation to osmotic fragility of cells.

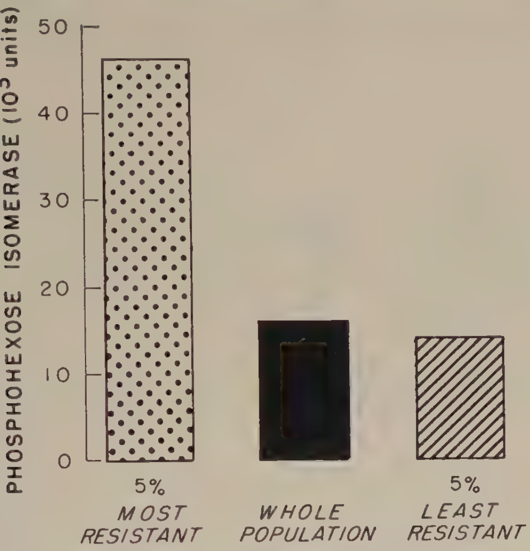

FIGURE 7. Erythrocyte phosphohexose isomerase activity in relation to osmotic fragility of cells.

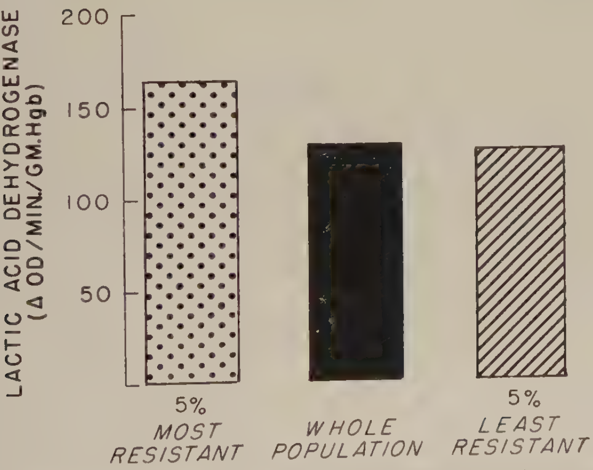

FIGURE 8. Erythrocyte lactic acid dehydrogenase activity in relation to osmotic fragility of cells.

changes accompanying *in vivo* aging? As summarized in TABLE I, both oxygen consumption measured in the presence of methylene blue and C^{14}-acetate incorporation into the total lipid and fatty acids were consistently greater in young than in old red cells. Erythrocytes employed in these studies were fractionated by the centrifugation method.

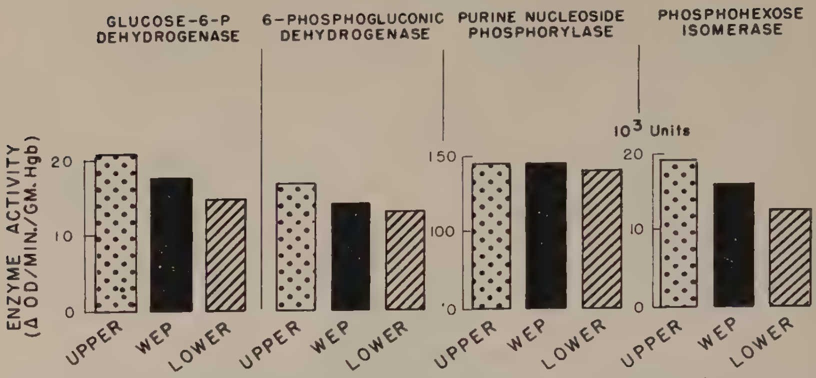

FIGURE 9. Erythrocyte enzyme activity in relation to the density of erythrocytes. WEP = whole erythrocyte population.

TABLE 1

ERYTHROCYTE OXYGEN CONSUMPTION AND LIPID SYNTHESIS

Fraction of erythrocytes*	Oxygen consumption†	C^{14}-acetate incorporation‡	
		Total lipids	Fatty acids
	µl./hr./gm. hgb.	*percentages*	
Upper (young)	580	0.31	0.25
Middle	450	0.14	0.10
Lower (old)	430	0.11	0.06

* Erythrocytes were fractionated by the method of centrifugation.[20]

† Erythrocyte oxygen consumption was measured under the following conditions: 30 µmoles of glucose, 0.25 µmoles methylene blue, 1.0 ml. of washed red blood cell suspension (hematocrit about 50) and sufficient Krebs-Ringer phosphate solution, *p*H 7.4, to make 3.0 ml. The center well contained 0.15 ml. of 6N NaOH.

‡ C^{14}-acetate incorporation studies were carried out under the following conditions: 30 µmoles of glucose; 1-C^{14}-acetate, 0.6 µc. (specific activity, 4.11 mc./mmole); 9 µmoles sodium versenate; 0.25 µmoles methylene blue, 1.0 ml. of washed red blood cell suspension (hematocrit about 50); and sufficient Krebs-Ringer phosphate solution, *p*H 7.4, to make 3.0 ml. Incubated for 2½ hours at 37° C. Total lipids were recovered from the erythrocytes and an aliquot hydrolyzed to fatty acids.[27] Both total lipids and fatty acids were counted under standard conditions.[28] The C^{14}-acetate incorporation is expressed in terms of percentage of added counts which were recovered in the total lipids and fatty acids.

Conclusions

The present studies lead to the following conclusions.

(1) The levels of at least 3 enzymes important in erythrocyte glucose metabolism, namely, glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphohexose isomerase are relatively high in young erythrocytes; these levels diminish markedly with the aging of the RBC *in vivo*.

(2) This striking decrease in enzyme activity does not involve all erythrocyte enzymes; purine nucleoside phosphorylase and lactic acid dehydrogenase are examples of enzymes that show relatively small decrements in activity.

(3) Alterations in certain metabolic functions such as glucose oxidation and lipid synthesis are associated with the changes in enzyme activity.

It seems reasonable to suggest that the diminution in activity of certain critical enzymes may be a determinant of the life span of the erythrocyte *in vivo*.

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AN HEREDITARY ENZYMATIC DEFECT IN RED BLOOD CELLS: ITS RELATION TO CERTAIN DRUG-INDUCED HEMOLYTIC ANEMIAS*

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Deficiency of the enzyme glucose-6-phosphate dehydrogenase in red blood cells results in detectable abnormalities in the metabolism of glutathione and in susceptibility to certain drug-induced hemolytic anemias. The relationship between G-6-PD activity and the metabolism of glutathione may be seen in the following scheme:† [1, 2]

$$\text{G-6-P} + \text{TPN}^+ \overset{\text{G-6-PD}}{\rightleftharpoons} \text{6-phosphogluconolactone} + \text{TPNH} + \text{H}^+ \quad (1)$$

$$\text{6-phosphogluconolactone} \xrightarrow{\text{lactonase}} \text{6-PG} \quad (2)$$

$$\text{6-PG} + \text{TPN}^+ \overset{\text{6-PGD}}{\rightleftharpoons} \text{ribulose-5-phosphate} + CO_2 + \text{TPNH} + \text{H}^+ \quad (3)$$

$$\text{GSSG} + \text{TPNH} + \text{H}^+ \overset{\text{GSSG red.}}{\rightleftharpoons} 2\ \text{GSH} + \text{TPN}^+ \quad (4)$$

Beutler[3] employed an *in vitro* test of gluthathione stability as a means of detecting individuals susceptible to hemolytic anemia caused by the ingestion of primaquine. The "sensitive" erythrocytes were identified by a loss of reduced glutathione after incubation with acetyl phenylhydrazine. Using this test to screen a random population, he estimated that approximately 10 per cent of Negroes, but only rare Caucasians, had drug-sensitive erythrocytes. Other workers have found GSH abnormalities in subjects with hemolytic anemias induced by naphthalene,[4] fava beans,[5] and nitrofurantoin.[6] Carson[7] detected a deficiency of the enzyme G-6-PD in the red blood cells of subjects sensitive to primaquine. An hereditary deficiency of erythrocyte G-6-PD occurs in subjects sensitive to fava beans and to naphthalene, in occasional individuals with hemolytic anemia of unknown cause, in healthy subjects without history of disease, and in the relatives of all of these groups.[8] This report concerns the mode of inheritance of the enzymatic defect and the relationship between measurements of erythrocyte G-6-PD and GSH stability.

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† The following abbreviations are used in this paper: G-6-PD, glucose-6-phosphate dehydrogenase; TPN, triphosphopyridine nucleotide; 6-PG, 6 phosphogluconate; 6-PG-P, 6 phosphogluconic dehydrogenase; TPNH, reduced triphosphopyridine nucleotide; GSSG, oxidized glutathione; GSSG red, glutathione reductase; GSH, reduced glutathione; and APH, acetyl phenylhydrazine.

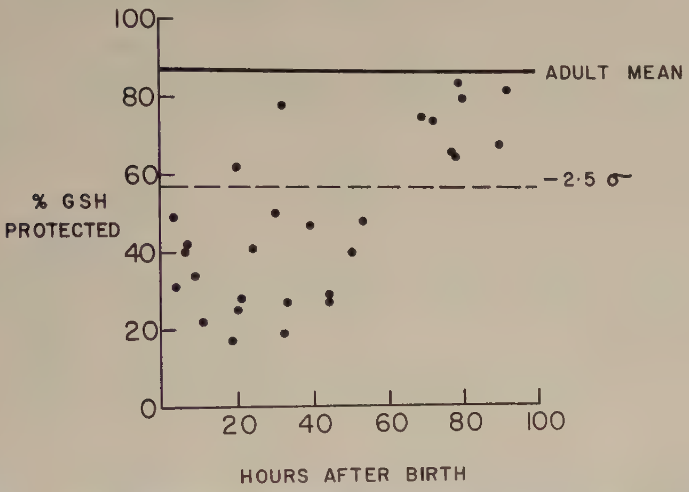

FIGURE 1. GSH stability in the newborn. Percentage of GSH protected equals percentage of reduced glutathione remaining after 2-hour incubation with acetyl phenylhydrazine.

Relationship Between Erythrocyte G-6-PD and GSH stability

The stability of GSH upon incubation with APH shows a high degree of correlation with the level of the enzyme G-6-PD. Three levels of enzyme activity (high, intermediate, and low) can be distinguished and are accompanied by comparable levels of GSH stability. In 123 children and adults the correlation coefficient between GSH stability and G-6-PD was +0.79 ($p = < 0.001$).[8] Newborn infants exhibit instability of GSH in their red cells.[9] This characteristic of the newborn disappears at approximately 95 hours of age (FIGURE 1). It is not related to deficiency of GSSG red. nor of G-6-PD: in fact, the levels of G-6-PD are higher than in adults.[10] Some other explanation must be sought for the GSH instability in this age group.

The Mode of Inheritance of G-6-PD Deficiency

A random population of approximately 150 Negroes and 150 Caucasians, none of whom had a history of hemolytic disease, was tested for the presence of erythrocyte G-6-PD. The enzyme was found to be deficient in approximately 8 per cent of the Negroes and 2 per cent of the Caucasians, an incidence similar to that reported by Beutler[3] for GSH instability. The enzymatic defect was also encountered in 5 subjects known to have experienced hemolytic anemia associated with ingestion of naphthalene, 4 following consumption of fava beans, 1 following sulfonamide administration, and 3 with hemolytic anemias of unknown cause. Among 140 relatives of all the affected subjects, 40 were found to have deficiency of the enzyme (FIGURE 2). Three generations were available for study in each of two families and the defect appeared in each generation. These data are in complete accord with previously reported

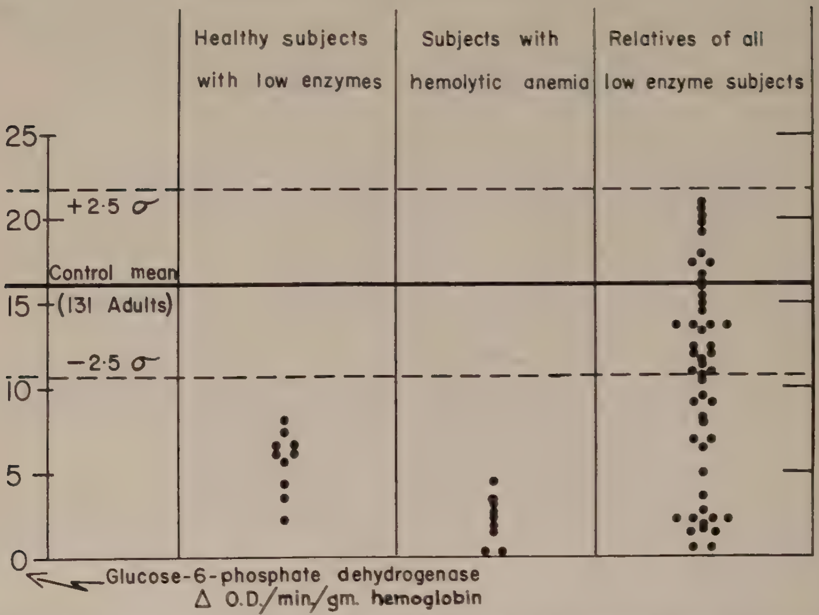

FIGURE 2. Enzyme levels in subjects with deficiency of erythrocyte glucose-6-phosphate dehydrogenase and their relatives. These data illustrate, but do not include, all of those presented in the text.

evidence[7,8] that an hereditary deficiency of G-6-PD is associated with certain drug-induced hemolytic anemias.

In 23 families studied, G-6-PD deficiency was present in at least 1 child and 1 parent. Male children were affected in 14 families and, in each instance, the genetic defect was traced to the mother. Among 37 affected male subjects studied only 6 were parents. The occurrence of the enzymatic defect mainly in male children and female parents, as well as the failure to trace the genetic defect in the male children to their fathers, suggests that sex linkage is the mode of inheritance. These findings are in accord with Childs' conclusions, which he based on GSH stability studies.[11]

The distribution of subjects into 2 groups characterized by low and intermediate enzyme levels (TABLE 1) reveals a preponderance of females and a

TABLE 1
DISTRIBUTION OF SUBJECTS WITH DEFICIENT G-6-PD
(G-6-PD is expressed as Δ O.D./gm. hgb./min.)

Subjects	Low enzyme (2.75 ± 2.0)*	Intermediate enzyme (8.9 ± 1.3)*
Males	35	2
Females	10	20

* Mean level of glucose-6-phosphate dehydrogenase ±1 standard deviation.

paucity of males in the intermediate group. Although the data are insufficient to be conclusive, they suggest that G-6-PD deficiency is controlled by a dominant gene, and that the phenotypic expression is modified in the female. The alternate possibility that the low enzyme levels represent the homozygous and the intermediate the heterozygous state was not borne out in the family studies.*

Summary

Deficiency of erythrocyte glucose-6-phosphate dehydrogenase is an hereditary defect associated with certain drug-induced hemolytic anemias.

A high degree of correlation is found between the level of G-6-PD and the *in vitro* stability of reduced glutathione except in the newborns in whom a transient instability of GSH exists despite high levels of enzyme.

The enzymatic deficiency appears to be related to a dominant gene, which is sex linked and sex modified.

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* In one family a mother with a very low level of G-6-PD produced 4 normal sons. In another family a normal father and an intermediate mother produced a daughter who was in the low group.

ORGANIZATION OF RED CELL GLYCOLYTIC ENZYMES: CELL COAT PHOSPHORUS TRANSFER*

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This discussion of human red cell metabolic organization is limited to problems connected with the uptake and elimination of inorganic phosphate.

In freshly drawn blood the red cells reveal an almost purely glycolytic metabolism; that is, glucose carbons terminate quantitatively in lactic acid with minimal routing through pentose phosphate. While glucose is being consumed at a constant rate, one finds an unchanging pattern of intermediate phosphorus metabolites inside the cell, including adenosine phosphates and a pool of inorganic phosphate. This equilibrium concentration is sufficient in the case of several of the intermediates to allow isolation in good purity. FIGURE 1 shows the typical pattern we have obtained by Dow-1 ion-exchange column chromatographic separation of phosphorus metabolites from the normally metabolizing human red cell.

When inorganic P^{32} orthophosphate was added to blood during this steady-state metabolism the phosphate compounds in the red cell became radioactive at different rates. In a 1-hour incubation experiment the relative specific activities (radioactivity per unit amount of phosphorus) shown in FIGURE 2 were observed. The rounded values refer to the following experimental determinations: first, adenylate, the 3 labile phosphoruses of adenosine diphosphate (ADP), and adenosine triphosphate (ATP); second, hexose, the 6 phosphoruses of the glucose and fructose monophosphates and diphosphates; and third, glycerate, the 3 phosphoruses of monophosphoglycerate (MPG) and the 2,3-diphosphoglycerate (2,3-DPG). The differences in specific activity between each group are large and easily reproducible experimentally. Small differences that were found within each group cannot be discussed here. The size of each box in the figure is roughly proportional to the phosphorus "pool" content.

The sequence of the phosphate reaction steps must follow the order of decreasing specific activities. The data show that a phosphorus atom from external inorganic phosphate passes through adenylate phosphorus before reaching internal inorganic phosphate, and we have concluded that there is little or no direct contact or exchange between the external and internal inorganic phosphates. The internal inorganic phosphate equilibrium level and its rate of labeling by P^{32} may be largely controlled by the phosphorylase reaction: glycogen + inorganic phosphate ↔ glucose-1-phosphate. We have preliminary evidence that a glycogen shunt is an integral part of normal red cell glycolysis.

Barring unforeseen mechanisms, it can be concluded from the general equation of glycolysis that the transfer of phosphorus from external inorganic

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* The work reported in this paper was supported in part by the San Diego Heart Association, San Diego, Calif.

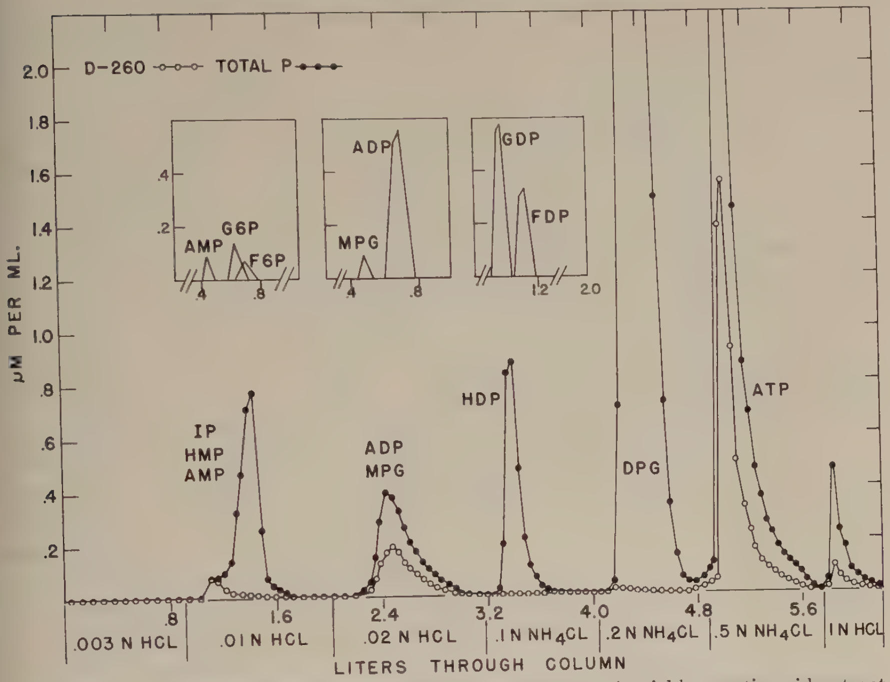

FIGURE 1. Isolation of red cell glycolytic intermediates. A trichloroacetic acid extract of 240 ml. of human red cells was chromatographed on a 1.5 × 30 cm. column of Dow-1 × 8 chloride, 60–80 wet mesh, at 5 ml. per min. with eluants as indicated. Inserts: linear gradient formate rechromatography of chloride peaks on 1 × 14 cm. columns of Dow-1 × 8 formate, 100–325 wet mesh, 1 ml. per min.; HMP, 2 liters of 0 → 1N formic acid after carbon removal of IP; ADP-MPG, 1 liter 0 → 1N *p*H 6.4 ammonium formate; GDP-FDP, 2 liters 0 → 1N *p*H 3.0 ammonium formate.

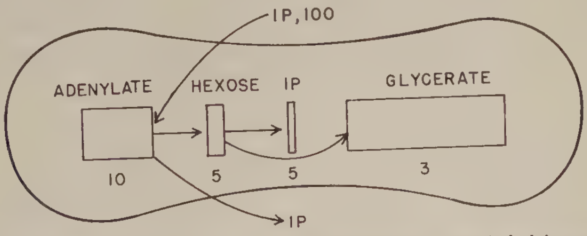

FIGURE 2. Movement of phosphorus from extracellular P^{32} labeled inorganic phosphate through phosphate pools of red cell.

phosphate to adenylate takes place via the carboxyl phosphate of 1,3-diphosphoglycerate (1,3-DPG) formed by glyceraldehyde-phosphate dehydrogenase, and that the reaction occurs in the cell coat: external IP* + glyceraldehyde-P → 1*,3-DPG → adenylate-P* + 3-PG. The normal red cell equilibrium level of the 1,3-DPG is so low or even nonexistent due to enzyme coupling) that it was not isolated for radioassay.

An alternate view could involve an unknown factor carrying inorganic phosphate across the cell coat. The literature is crowded with unidentified carriers; moreover, such an agent would still be required to transfer external inorganic phosphate specifically to glyceraldehyde-phosphate dehydrogenase without leaving any P^{32} trace of itself.

Some definitions may help to clarify the discussion. We have already referred to the cell coat, a term that is applied to a layer at the periphery of the cell. This coat has a thickness probably variable within any one cell of some 50 to 250 Å, and consists of a framework of insoluble fibrous protein and lipin,[1] containing its own cytoplasm and separated from the interior of the cell and from the plasma by phase boundaries that will be called inner and outer surfaces of the coat.

Let us say, then, that the glyceraldehyde-phosphate dehydrogenase is located within the cell coat and that movement of the cytoplasm makes possible frequent contact of the enzyme with both outer and inner surfaces of the coat. It should not stretch the imagination unduly to have the enzymes in the cell coat in a more active motion than that due to molecular collision. The property of movement in living cellular cytoplasm is so universal, where it can be seen, that it would be reasonable to assume its validity for the red cell until proved otherwise.

The unique characteristic of the glyceraldehyde-phosphate dehydrogenase is that its specific surface catalytic site possesses a high affinity for inorganic phosphate, diphosphopyridine nucleotide (DPN), and glyceraldehyde-phosphate, which it transforms into reduced DPN (DPNH) and 1,3-diphosphoglycerate.

Figure 3 illustrates the model in more detail. The enzymes lactic dehydrogenase and 3-phosphoglycerate kinase have been directly coupled to the glyceraldehyde-phosphate dehydrogenase in the cell coat. Starting with an empty catalytic site on a molecule of glyceraldehyde-phosphate dehydrogenase, we have the enzyme turn through the cytoplasm, picking up in succession a molecule of glyceraldehyde-phosphate from the inner surface (or perhaps more specifically from triose-phosphate isomerase), inorganic phosphate from the outer surface, and DPN from the lactic dehydrogenase. At this instant the

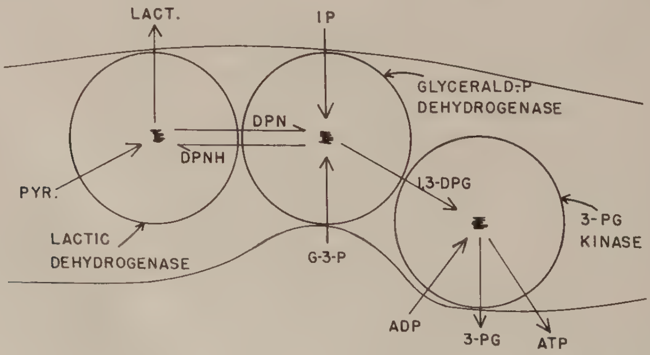

Figure 3. Mechanism for the transfer of phosphorus from extracellular inorganic phosphate into red cell phosphates.

three react, the product DPNH returning immediately to the lactic dehydrogenase and the 1,3-DPG finding a site of higher affinity on the 3-phosphoglycerate kinase enzyme.

The model attempts to solve one of the principal organizational problems posed by the experimental data; that is, how to incorporate phosphorus without admitting any free inorganic phosphate into the cell, at the same time not losing any of the phosphorylated intermediates to the outside.

As already emphasized, during uniform metabolism, phosphorus enters the cell and, with the total cellular phosphorus remaining constant, must leave at the same rate. The only net phosphorus synthesis of the accepted equation of glycolysis is ATP formation. Since the amount of ATP does not change, its hydrolysis is most probably the immediate source of the phosphorus that is leaving the cell. Two reactions might at first appear plausible: first, the splitting of ATP to give internal inorganic phosphate which, by some second reaction or free diffusion, moves out of the cell; second, the hydrolysis of ATP in the cell coat, with the direct elimination of the resulting inorganic phosphate from the cell. Because of the relatively low specific activity of the internal inorganic phosphate, evidence against free diffusion, and a wish to avoid carriers, we prefer the second process.

Our model, therefore (FIGURE 4), places an ATP hydrolysis enzyme in the red cell coat capable of contacting both surfaces. Its catalytic site has a high affinity for ATP, which it collects from the interior cytoplasm. The problem is to have the protein release its two products, inorganic phosphate and ADP, in different directions. We propose that the rate of reaction is sufficiently more rapid than enzyme motion to insure ADP dropping inside immediately after ATP contact. We assume that the inorganic phosphate remains lightly bound to the enzyme until it finds a better gradient to the exterior; however, this is admittedly a weak argument.

There is one more product of the ATP splitting reaction; namely, energy. To complete our scheme we postulate that the principal use of this energy is to impart vigorous motion to the cytoplasm.

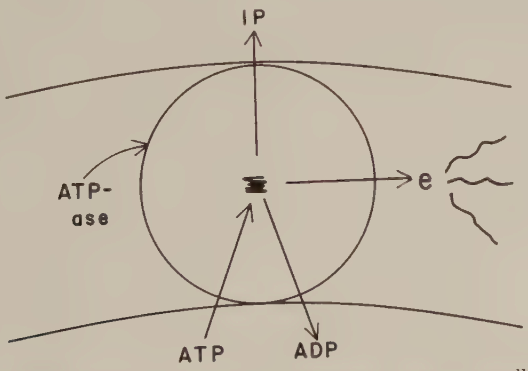

FIGURE 4. Mechanism for the transfer of phosphorus from intracellular organic phosphates to extracellular inorganic phosphate.

The reader may question the value of such speculation when it would appear simple to test for specific enzyme localizations, using that much-studied red cell coat derivative, stroma or "ghost." It is felt that lessons learned from the exhaustive research in progress on the localization of biochemical factors and processes within mitochondria[2] make it clear that the ghost is inadequate for our purposes and that new methods are required to produce derived coats that are more representative of the original native state.

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ENZYMES OF PORPHYRIN SYNTHESIS IN RED BLOOD CELLS

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The enzymes in erythrocytes that synthesize protoporphyrin may conveniently be divided into three groups. The first group converts unknown derivatives of glycine and succinate to δ-aminolevulinic acid. This group includes enzymes bound to cell particulates. The second group converts δ-aminolevulinic acid to coproporphyrinogen and is soluble. The third group converts coproporphyrinogen to protoporphyrin and, although it is found in the easily sedimentable portion of the cell debris following freeze-thawing, it may be "solubilized" by grinding in a colloid mill.

The first group was studied with both washed, intact chicken red cells and with cells hemolyzed by freeze-thawing. Porphyrin formation was measured spectrophotometrically and the formation of δ-aminolevulinic acid separated from the remainder of the sequence by observing the relative effect of the inhibitors on the porphyrin obtained first with glycine, then with δ-aminolevulinic acid as substrate. Details of this and of other results mentioned in this paper will be published elsewhere.[1]

Our results confirm and extend the work of other investigators and add a few new facts. The synthesis of δ-aminolevulinic acid from glycine with intact red cells seems to require, first, an electron transfer system to oxygen (inhibition by CO, anaerobiosis);[2] second, oxidative phosphorylation (inhibition by dinitrophenol;[2] third, a citric acid cycle (inhibition by malonate, trans-aconitate, fluoroacetate, or arsenite, or a slight accelerating effect of citric acid cycle members;[2-4] fourth, pyridoxal-phosphate (inhibition by desoxypyridoxamine and isonicotinic hydrazid, overcome by pyridoxal phosphate);[5] and, finally, glutamine (that is, inhibition by azaserine, overcome by glutamine). The last of these reactions is shown in FIGURE 1.

Since the citric acid cycle, the cytochromes, and the oxidative phosphorylation systems are known to be present in mitochondria, and since this first group of enzymes contains these systems and is particulate, it is tempting to conclude that δ-aminolevulinic acid is synthesized in connection with mitochondria.

Most of the effects observed on the synthesis of δ-aminolevulinic acid are quite indirect; however, the requirement for pyridoxal-phosphate is easily explained as the formation of a Schiff base with glycine, thus activating the methylene group to condense with some derivative of succinic acid to yield α-amino-β-ketoadipic acid.

The amino acids L-cysteine, L-serine, L-arginine, L-proline and L-alanine were found to inhibit the synthesis of δ-aminolevulinic acid (TABLE 1); L-aspartate, L-asparagine, L-glutamate, L-threonine, and D,L-valine were not inhibitory. A possible explanation is the existence of a coupling between globin and δ-aminolevulinic acid synthesis. It is conceivable that in erythro-

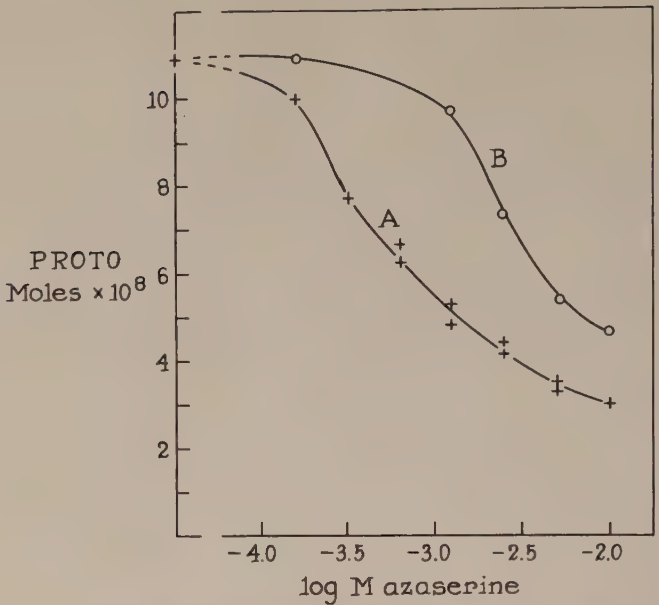

FIGURE 1. The inhibition of protoporphyrin (PROTO) synthesis in washed chicken red cells by azaserine and its suppression by glutamine. Curve A, 0.07 M glycine plus 0.01 M α-ketoglutarate plus azaserine at indicated concentrations; Curve B, same as Curve A, but with 0.01 M glutamine added.

TABLE 1
INHIBITION OF δ-AMINOLEVULINIC ACID SYNTHESIS BY CERTAIN AMINO ACIDS*

Amino acid	Concentration (M)	Per cent yield of protoporphyrin
—	—	100
1-Cysteine	0.035	5
	0.0087	43
	0.0043	75
1-Serine	0.035	52
	0.017	75
	0.0087	85
1-Arginine	0.035	56
1-Proline	0.035	64
	0.017	80
1-Alanine	0.035	70
	0.017	79

* Substrates: 0.035 M glycine and 0.01 M α-ketoglutarate. Concentrations of amino acids are shown in the center column.

blasts during rapid globin synthesis the concentration of such inhibitory amino acids might be lowered and thus allow synthesis of δ-aminolevulinic acid, and thereby of heme, to take place. Likewise, in certain mutants of the alga *Chlorella* and in a strain of the protozoan *Tetrahymena*, both of which synthesize protoporphyrin, the pigment accumulates most rapidly at the end of the phase of rapid cell multiplication when the nitrogen supply of the medium has been depleted.

The slight activity of frozen and thawed chicken erythrocytes in forming protoporphyrin from glycine is enhanced by the simultaneous presence of a large number of compounds. The order of additions that gives progressive increases in protoporphyrin to 35 per cent of that of the intact cell is as follows: glycine, pyridoxal-phosphate, coenzyme A, diphosphopyridine nucleotide (DPN), glutamine, and inosine. The addition of various other cofactors raised the yield to 60 per cent of that of the intact cell. A pig liver extract, absorbed on Nuchar and eluted at *p*H 4 or 5, increased the yield to 80 per cent. Members of the citric acid cycle that enhance the yield in intact cells have no effect on hemolyzed cells.

The substances which must be added to reactivate synthesis of δ-aminolevulinic acid in these hemolyzed red cells are presumably those that leak out and become limiting. If δ-aminolevulinic acid synthesis is taken as a measure of the "health" of a red cell, these additives may be of value in the preservation of red cells in blood banks.

The second soluble group of enzymes that converts δ-aminolevulinic acid to coproporphyrinogen (FIGURE 2) has been separated by zone electrophoresis on starch into three fractions: one condenses δ-aminolevulinic acid to porphobilinogen, the second condenses porphobilinogen to uroporphyrinogen isomer III, and the third decarboxylates uroporphyrinogen, forming coproporphyrinogen (FIGURE 3). This has been done with extracts from red cells of the chicken, the rabbit, and the fetal human; the order of migration varies with the species. Most of the experiments were done with the rabbit preparation.

Kinetic and inhibitor studies on the first fraction indicate that the enzyme specifically binds both molecules of δ-aminolevulinic acid required to form porphobilinogen. This conclusion is based on the observation of simple Michaelis-Menten-type kinetics, and the finding that no mixed pyrroles are formed from δ-aminolevulinic acid and methyl-δ-aminolevulinate or amino acetone. This enzyme has also been studied by Gibson *et al.*[6] and by Schmid and Shemin.[7]

The second fraction, which condenses porphobilinogen to uroporphyrinogen isomer III (FIGURE 2) was found to resemble a similar preparation of *Chlorella* extracts[8] in that heating it caused uroporphyrinogen isomer I to form instead of isomer III. Uroporphyrinogen isomer I is not converted to isomer III by the original unheated preparation, and therefore the heat-labile component must act at some stage preceding the closure of the macro ring. Bogorad has reported studies on similar preparations from spinach and from *Chlorella*,[9] and Lockwood and Rimington similarly on a preparation from chicken erythrocytes.[10] Since large amounts of uroporphyrin isomer I and coproporphyrin isomer I are excreted in the disease congenital porphyria, a low activity of this heat-labile component relative to the others in the biosynthetic sequence would explain the observed disorder, in part.

The third fraction decarboxylates the acetic acid side chains of uroporphyrinogen to form coproporphyrinogen (FIGURE 2). There is but little

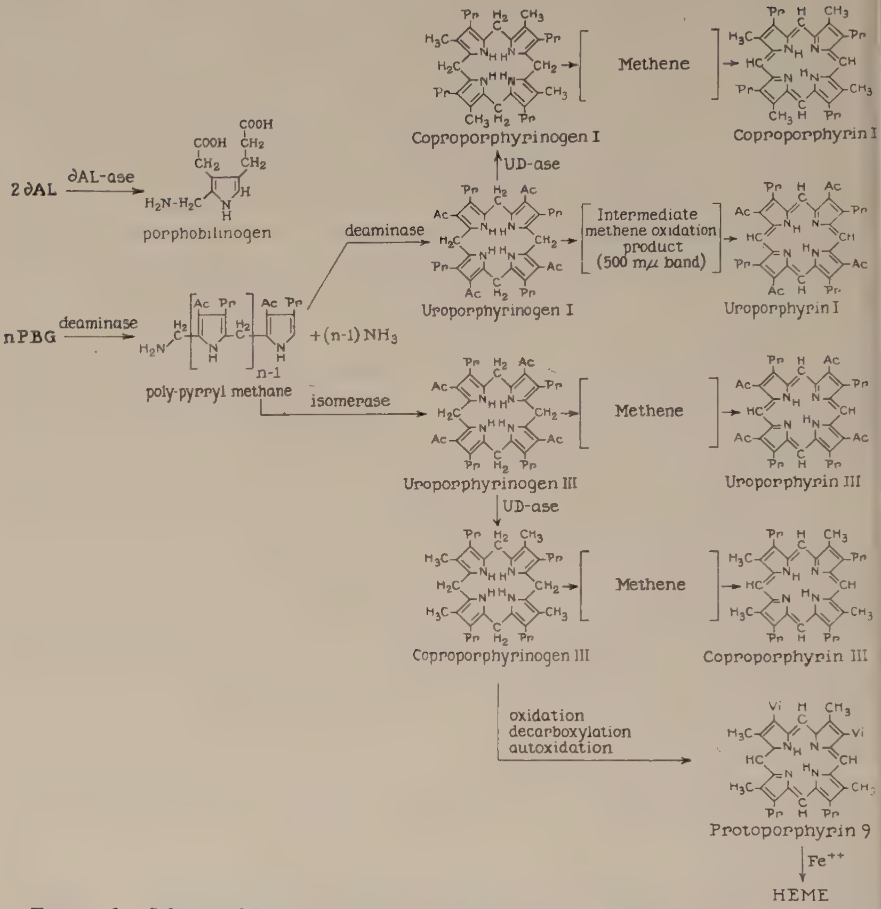

FIGURE 2. Scheme of heme biosynthesis in the red cell, starting with δ-aminolevulinic acid. Ac = $—CH_2CO_2H$; Pr = $—CH_2CH_2CO_2H$; Vi = $—CH{=}CH_2$; δAL-ase = enzyme that condenses δ-aminolevulinic acid to porphobilinogen. UD-ase = enzyme that decarboxylates uroporphyrinogen to form coproporphyrinogen.

specificity as to the isomers, uroporphyrinogen isomer I being decarboxylated at about one half the rate of isomer III. The colorless hexahydroporphyrin or porphyrinogen is the actual substrate for this enzyme; the colored uroporphyrin itself is not decarboxylated (TABLE 2). These facts may explain why both uroporphyrin and coproporphyrin of either isomer I or III may accumulate in the various porphyria diseases, assuming that the enzymes in human cells have similar properties. The amount of these porphyrins that form will be a function not only of the relative activity of the various enzymes in the sequence, but also of the presence of catalysts (for example, light) or of inhibitors (such as sulfhydryl groups) of the autoxidation of the porphyrinogens to the enzymatically inactive porphyrins. Bogorad has obtained small amounts of coproporphyrin on incubating reduced uroporphyrin with a prep-

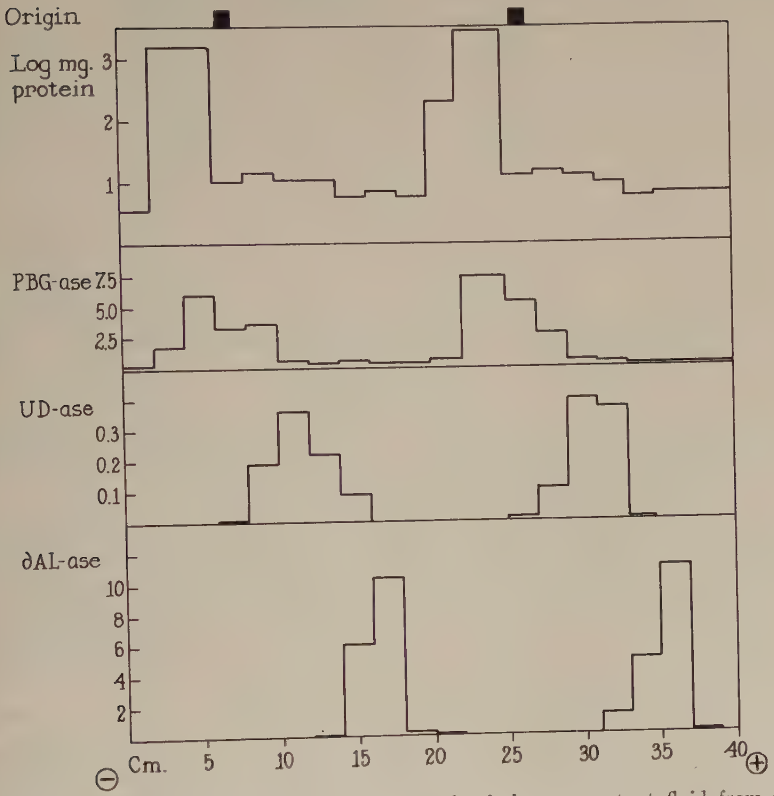

FIGURE 3. Electrophoretic separation on starch of the supernatant fluid from rabbit reticulocytes. The figures represent the total protein and total activity of eluates from 2-cm. wide segments of 2 identical starch plates. Two streaks were applied to the plate at "origin". δAL-ase activity is in μmoles increase in porphobilinogen (PBG); PBG-ase activity is in μmoles decrease of PBG; and UD-ase activity is in μmoles of coproporphyrinogen formed, all per hour per fraction.

TABLE 2

ENZYME PLUS UROPORPHYRINOGEN I AND III AND UROPORPHYRIN III*

Substrate	μMoles	μMoles isolated	
		URO	COPRO
Uroporphyrinogen I	340	130	52
Uroporphyrinogen III	400	38	210
Uroporphyrin III	400	316	0.6
Uroporphyrinogen III, no enzyme	400	287	0.7

* The solutions were incubated anaerobically for 16 hours at 38° C., then titrated with iodine to oxidize the reduced porphyrins.[1]

TABLE 3

SYNTHESIS OF PROTOPORPHYRIN FROM COPROPORPHYRINOGEN ISOMER III AND I IN HEMOLYZED CHICKEN RED CELLS*

Time incubation: hours	Protoporphyrin, μMoles		
	No coproporphyrinogen	Coproporphyrinogen III	Coproporphyrinogen I
0	—	7	7
1	3	42	18
4	3	91	18
18	9	113	21

* The frozen-thawed cells (2 ml.) were incubated for the indicated time with and without 140 μmoles of coproporphyrinogen at 38° C. The coproporphyrinogen isomer I contained ~10 per cent isomer III.

aration from *Chlorella*,[11] and Neve *et al.* have found that adding reduced uroporphyrin to hemolysed duck red cells increased the incorporation of Fe^{59} into heme.[12]

The third group of enzymes converts coproporphyrinogen isomer III to protoporphyrin. These preparations have no action either on coproporphyrin itself or on coproporphyrinogen isomer I (TABLE 3). The specificity as to isomers may explain the fact that only protoporphyrin isomer IX is found in nature, since it is derived from the isomer III series of coproporphyrin. The formation of the vinyl groups from the propionic acid side chains requires oxygen, but is not inhibited by cyanide ion.

The reactions catalyzed by the second and third groups of enzymes are schematized in FIGURE 3. Direct proof for all of these compounds except the poly-pyrryl methane has been obtained under various conditions. The normal pathway is δ-aminolevulinic acid → porphobilinogen → uroporphyrinogen III → coproporphyrinogen III → protoporphyrin IX and/or heme. Under abnormal conditions porphobilinogen first may condense to form uroporphyrinogen I and, therefrom, coproporphyrinogen I, and second, these reduced porphyrins may be autoxidized to the enzymatically inactive porphyrins.

Since the porphyrinogens cannot bind metals, the incorporation of the metal to form heme (or chlorophyll) must take place during or after the autoxidation to porphyrins. Since ferrous ion complexes rather readily with monomolecularly dispersed porphyrins, a kinetic study would be required to decide whether the complexing of ferrous ion is more rapid during or after the autoxidation.

Summary

The enzymes in erythrocytes that synthesize protoporphyrin may be divided conveniently into three groups. The first group converts active glycine and active succinate to δ-aminolevulinic acid. It requires cell particulates, oxidative phosphorylation, and a citric acid cycle. Certain amino acids inhibit this stage of the synthesis. The second group of enzymes is soluble and converts δ-aminolevulinic acid to coproporphyrinogen. Three colorless fractions have been separated by zone electrophoresis. One converts δ-

aminolevulinic acid to porphobilinogen, another converts porphobilinogen o uroporphyrinogen III, and the third converts uroporphyrinogen to coproporphyrinogen. The third group of enzymes converts coproporphyrinogen III (but not I) to protoporphyrin. Characteristics of these enzymes are discussed in relation to their function in the cell.

Acknowledgments

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ENZYMATIC SYNTHESIS OF δ-AMINOLEVULINIC ACID*

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It has been demonstrated that δ-aminolevulinic acid (δ-ALAC) is an intermediate in the synthesis of porphyrins[1, 2] and for the porphyrin-like moiety of vitamin B_{12}.[3] This aminoketonic acid arises biologically from the condensation of "active" succinate[4] and glycine (REACTION 1).

$$\begin{aligned} &HOOC{-}CH_2{-}CH_2{-}COOH + NH_2{-}CH_2{-}COOH \rightarrow \\ &\qquad HOOC{-}CH_2{-}CH_2{-}CO{-}CH_2{-}NH_2 + CO_2 \end{aligned} \tag{1}$$

However, the details of this condensation and the mechanism were not known.

This communication reports our finding that particle-free extracts of two photosynthetic bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum*, are capable of catalyzing the net synthesis of δ-aminolevulinic acid from succinate and glycine on addition of cofactors, including coenzyme A, adenosine triphosphate (ATP), pyridoxal phosphate, and Mg^{++} ions, or from succinyl-coenzyme A and glycine on addition of pyridoxal phosphate.[5] Laver and Neuberger[6] and Gibson *et al.*[7] recently reported that a particulate fraction obtained from erythrocytes of chickens treated with phenylhydrazine was capable of synthesizing this compound from α-ketoglutarate and glycine and from succinylcoenzyme A and glycine.

Lascelles[8] has demonstrated that not only are porphyrins synthesized by *R. spheroides* by the sequence of events previously elucidated in other cells, but also that porphyrins and porphobilinogen accumulate in the medium. We were encouraged to investigate this bacterium for the enzymes responsible for the synthesis of δ-aminolevulinic acid, for we found that this acid was also present in the medium after the cells were suspended in medium I of Lascelles. *R. spheroides* and R. *rubrum* were grown under anaerobic conditions in the light. The bacteria were harvested by centrifugation and washed with saline, and then 0.1 M phosphate buffer extracts of the broken cells were centrifuged at 105,000 g for different lengths of time. It can be seen in TABLES 1 and 2 that the extracts of both *R. spheroides* and *R. rubrum* synthesized δ-aminolevulinic acid from succinate and glycine and that there is a marked dependency for pyridoxal phosphate, CoA, ATP and Mg^{++} ions especially in the dialyzed extracts. It also can be seen (compare TABLES 1 and 2) that whereas extracts of *R. spheroides* obtained on centrifugation for 30 min. synthesize a fair amount of porphyrin from the formed δ-aminolevulinic acid, little synthesis occurred in those extracts obtained on a 3-hour centrifugation. The crude extracts of *R. rubrum* are not as dependent on the addition of CoA as are those of *R. spheroides*. However, it can be seen in TABLE 3 that extracts

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TABLE 1

ENZYMATIC SYNTHESIS OF δ-AMINOLEVULINIC (δ-ALAC) ACID IN *R. SPHEROIDES* EXTRACTS (30 MINUTES CENTRIFUGATION AT 105,000 g)

Incubation mixture	μMoles of δ-ALAC acid formed/mg. protein/60 minutes: Free δ-ALAC	δ-ALAC as porphyrin	Total δ-ALAC
1 *R. spheroides*			
Complete system	0.070	0.057	0.127
Minus pyridoxal phosphate	0.020	0.007	0.027
Minus CoA	0.006	0.000	0.006
Minus adenosine triphosphate	0.031	0.022	0.053
Minus ATP, plus adenosine diphosphate	0.040	0.052	0.092
Minus $MgCl_2$	0.085	0.067	0.152
α-Ketoglutarate in place of succinate	0.001	0.000	0.001
2 *R. rubrum*			
Complete system	0.054	0.007	0.061
Minus CoA	0.056	0.006	0.062

The complete system contained 50 μmoles succinate, 50 μmoles glycine, 1 μmole CoA, 0.25 μmoles pyridoxal phosphate, 5 μmoles ATP, 5 μmoles $MgCl_2$, 1.0 ml., 0.1 M phosphate buffer, *p*H 6.9 and 7.2 mg. protein of *R. spheroides* and 12 mg. protein *R. rubrum*. Total volume 2.5 ml.

TABLE 2

SYNTHESIS OF δ-ALAC IN EXTRACTS OBTAINED AFTER 3 HOURS' CENTRIFUGATION AT 105,000 g AND DIALYZED FOR 18 HOURS

Incubation mixture	μMoles of δ-ALAC formed/mg. protein/60 min.: Free δ-ALAC		δ-ALAC as porphyrin		Total δ-ALAC	
	R. spheroides	*R. rubrum*	*R. spheroides*	*R. rubrum*	*R. spheroides*	*R. rubrum*
Complete system	0.140	0.023	0.009	0.001	0.149	0.024
Minus pyridoxal phosphate	0.008	0.001	0.002	0.000	0.010	0.001
Minus CoA	0.026	0.012	0.001	0.001	0.027	0.013
Minus ATP	0.002	0.001	0.000	0.001	0.002	0.002
Minus $MgCl_2$	0.062	0.018	0.016	0.001	0.078	0.019

The incubation mixtures were similar to those in TABLE 1, except that 5.6 mg. of *R. spheroides* protein and 9.0 mg. of *R. rubrum* protein were used.

of *R. rubrum* which were treated with Dowex-1 have a marked dependency on the addition of CoA for synthesis of δ-aminolevulinic acid.

The free δ-aminolevulinic acid was determined by the method of Schuster[9] and was also identified by paper chromatography. In order to document further the identity of the product synthesized from succinate and glycine, the formed δ-aminolevulinic acid was isolated as the 2-methyl-3-carbethoxy-4-(3-propionic) pyrrole derivative[10] synthesized from either glycine-2-C^{14} or succinate-1-C^{14}. The formed δ-aminolevulinic acid was isolated as the

TABLE 3

EFFECT OF DOWEX-1 TREATMENT ON THE CoA REQUIREMENT OF *R. RUBRUM* ENZYMES

Incubation mixture	μMoles of free δ-ALAC/mg. portein/60 min.	
	Before Dowex-1 treatment	After Dowex-1 treatment
Complete system	0.023	0.022
Minus CoA	0.012	0.002

Reaction conditions similar to TABLES 1 and 2. Extract contained 7.4 mg. protein.

TABLE 4

SYNTHESIS OF δ-AMINOLEVULINIC ACID

Radioactive substrate	Radioactivity of δ-ALAC derivative [2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole]	
	Carrier δ-ALAC added	
	Before incubation	After incubation*
	cpm	*cpm*
Succinate-1-C^{14}	8	833
Glycine-2-C^{14}	39	873

The incubation mixture was similar to those in previous tables, except that the substrates were radioactive (0.15 mc./mmole). One mmole of carrier δ-ALAC was added.

* 1 μmole of δ-ALAC was formed in these flasks.

derivative after the addition to the incubation mixture of nonradioactive δ-aminolevulinic acid. An extract of *R. spheroides* was incubated with all the necessary components in which the glycine or succinate was radioactive. It can be seen from TABLE 4 that the derivative of δ-aminolevulinic acid isolated from the incubation mixture, to which the carrier δ-aminolevulinic acid was added after the incubation period, was highly radioactive and that the radioactivity of the sample synthesized from radioactive succinate was equal to that synthesized from the radioactive glycine. The equality of radioactivity demonstrates experimentally that one mole of succinate condenses with one mole of glycine (REACTION 1). The radioactivity found agreed with the value calculated from the determined amount synthesized and the amount of carrier δ-aminolevulinic acid added. It may also be seen from TABLE 4 that the addition of carrier δ-aminolevulinic acid at the beginning of the incubation period inhibited its enzymatic formation.

It can be surmised from the foregoing that the formation of δ-aminolevulinic acid can be divided into two over-all reactions: first, the activation of succinate by CoA and ATP, followed by condensation of "active" succinate with a pyridoxal derivative of glycine. Evidence that the extracts of *R. spheroides* and *R. rubrum* contain activating systems for succinate that require, CoA, ATP, and Mg^{++} ions can be gathered from TABLE 5, which represents experiments on the formation of hydroxamic acid. This table also shows that the

TABLE 5

HYDROXAMIC ACID FORMATION

Incubation mixture	μMoles of hydroxamic acid formed/mg. protein/60 min.	
	R. spheroides	*R. rubrum*
Complete system	0.590	0.340
Minus $MgCl_2$	0.130	0.045
Minus CoA	0.090	0.033
Minus ATP	0.118	0.003
Minus CoA and ATP	0.109	0.009
Minus succinate	0.030	0.000

The complete systems contained 50 μmoles succinate, 1 μmole CoA, 5 μmoles ATP, 5 μmoles $MgCl_2$, 0.7 ml. of 2.8 M hydroxylamine, 1.0 ml. of 0.1 M phosphate buffer (*p*H 6.9), 3.3 mg. of *R. spheroides* protein, and 9 mg. of *R. rubrum* protein.

TABLE 6

EFFECT OF LIGHT AND ANAEROBIOSIS ON δ-ALAC FORMATION

	Air		N_2	
	Light	Dark	Light	Dark
μMoles of free δ-aminolevulinic acid formed per 60 min.	0.73	0.39	0.23	0.18

The *R. spheroides* extract was the supernatant fluid obtained on centrifugation for 30 min. at 20,000 g. The incubation mixture contained 100 μmoles succinate, 100 μmoles glycine, 0.5 μmoles pyridoxal phosphate, 2.5 μmoles CoA, 10 μmoles ADP, 10 μmoles $MgCl_2$, 1.3 ml. of 0.1 M phosphate buffer (*p*H 6.9), and 36 mg. of protein. Final volume 4.0 ml.

hydroxamic acid of succinate is formed from succinate on addition of these cofactors.

The formation of δ-aminolevulinic acid in a particulate-containing extract (centrifuged for 30 min. at 20,000 g) is greater in the light than in the dark, as shown in TABLE 6. In this experiment the nucleotide added was adenosine diphosphate (ADP), and presumably the light effect is due to photophosphorylation of the ADP to ATP.

The "active" succinate appears to be succinylcoenzyme A. This is supported by the experiments summarized in FIGURE 1 and in TABLE 7. FIGURE 1 shows that the rate of synthesis of δ-aminolevulinic acid is far greater from succinylcoenzyme A than from the components (succinate and CoA) even on addition of ATP. In TABLE 7, it can be seen that succinylcoenzyme A can substitute for succinate, CoA, and ATP and that little synthesis occurs from succinate plus CoA. It can also be seen in TABLE 7 that no δ-aminolevulinic acid is formed from succinylcoenzyme A and glycine on omission of pyridoxal phosphate.

Further experimental evidence that 2 over-all reactions are concerned with the formation of δ-aminolevulinic acid and that these enzymatic activities can be separated is presented in TABLE 8. The extract of *R. spheroides* was

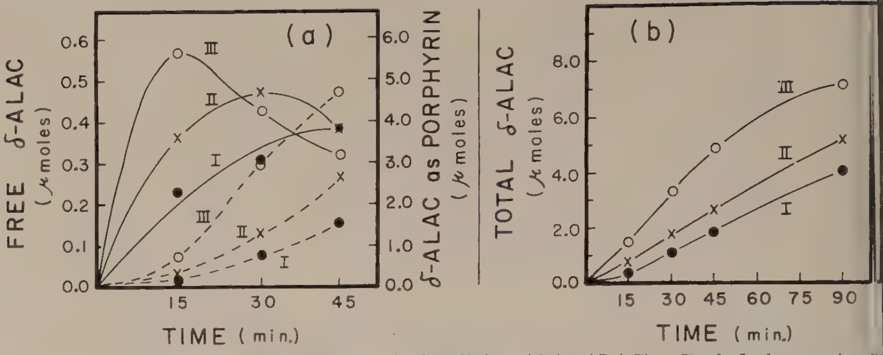

Figure 1. Rates of synthesis of δ-aminolevulinic acid (δ-ALAC). Each flask contained 150 µmoles glycine, 15 µmoles $MgCl_2$, 0.75 µmoles pyridoxal phosphate, 4.5 ml. of 0.1 M phosphate buffer (*p*H 6.9), and 3.0 ml. of extract (80.2 mg. protein). In addition, flask I contained 15 µmoles succinate and 15 µmoles CoA; flask II continued 15 µmoles succinate, 15 µmoles CoA, and 5 µmoles ATP, and flask III contained 15 µmoles succinyl-coenzyme A. Final volume 9 ml. (a) The solid lines represent determination of free δ-aminolevulinic acid; the broken lines represent the amount of the compound as porphyrin. (b) The lines represent the total amounts of δ-aminolevulinic acid.

Table 7
Rate of Synthesis of δ-ALAC from Different Substrates with *R. rubrum* Enzymes

Added components	µMoles of δ-ALAC formed after 20 min. of reaction	
	Before Dowex-1 treatment	After Dowex-1 treatment
Succinate + CoA + ATP.......	0.064	0.052
Succinate + ATP..............	0.022	0.007
Succinate + CoA..............	0.003	0.001
Succinyl CoA..................	0.060	0.052
Succinyl CoA minus $MgCl_2$......	0.066	
Succinyl CoA minus pyridoxal-p.	0.000	

The incubation system contained 50 µmoles glycine, 0.25 µmoles pyridoxal phosphate, 2 µmoles $MgCl_2$, and 8 mg. of protein. To this incubation mixture the following were added as shown in the first column, 2 µmoles succinate, 2 µmoles CoA, 2 µmoles ATP, and 2 µmoles succinylcoenzyme A. Total volume, 2 ml.

Table 8
Distribution of the Enzymatic Activities Obtained on Ammonium Sulfate Fraction

Per cent saturated $(NH_4)_2SO_4$	µMoles of the products formed/mg. protein/20 min.	
	δ-ALAC from succinyl CoA	Hydroxamic acid from succinate
25 to 35 per cent fraction.......	0.05	0.025
45 to 65 per cent fraction.......	0.01	0.360
Before fractionation............	0.05	0.300

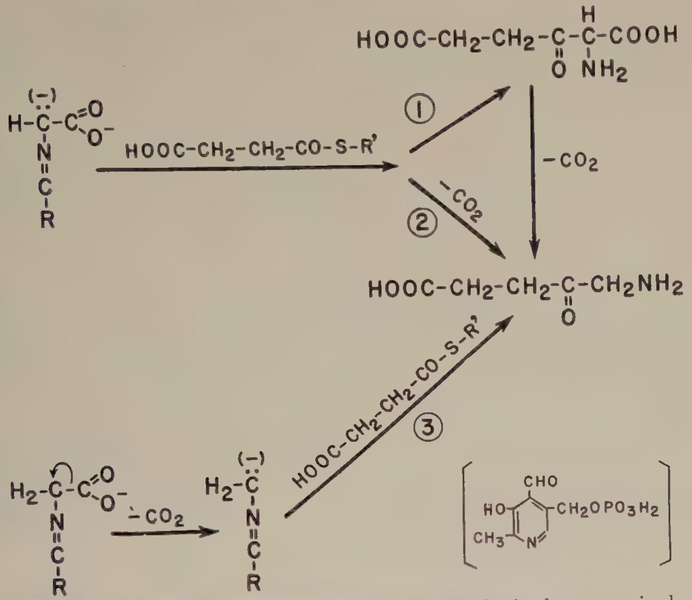

FIGURE 2. Mechanisms of δ-aminolevulinic acid synthesis from succinylcoenzyme A and pyridoxal phosphate derivative of glycine.

TABLE 9

DECARBOXYLATION OF GLYCINE-1-C^{14}

Effect of Succinate*

	Radioactivity of CO_2
	cpm
Complete system	203
Minus succinate	5

Parallelism of $C^{14}O_2$ Formation and δ-ALAC Formation†

Time of incubation	Total δ-ALAC formed	Radioactivity of CO_2
	μmoles	*cpm*
40	1.22	555
80	2.08	791
120	2.40	925

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* The reaction mixture is similar to that of TABLE 1. The extract of *R. spheroides* was that obtained on centrifugation for 3 hours at 105,000 g and then dialyzed overnight. The amount of protein used was 3.3 mg., and the glycine was labeled in its carboxyl group.

† An *R. spheroides* extract, centrifuged at 105,000 g for 30 min., containing 40 mg. protein, was incubated with 50 μmoles succinate, 50 μmoles glycine 1-C^{14}, 2.5 μmoles CoA, 0.5 μmoles pyridoxal phosphate, 10 μmoles ATP, and 10 μmoles $MgCl_2$; final volume 4 ml. The radioactivity of the glycine-1-C^{14} was 0.028 mc./mole.

fractionated with ammonium sulfate. The 25 to 35 per cent fraction contains most of the enzymatic system concerned with the condensation of succinylcoenzyme A with the pyridoxal phosphate derivative of glycine, whereas the 45 to 65 per cent fraction contains the succinate activating system.

It would appear reasonable to conclude from the cofactor requirements that succinylcoenzyme A condenses with a stabilized carbanion formed from glycine and pyridoxal phosphate. This stabilized anion can be formed either by a loss of a proton or by the loss of CO_2, as indicated in FIGURE 2. It would appear from TABLE 9, in which glycine-1-C^{14} was included in the incubation mixture, that the stablized carbanion is formed by the loss of a proton, for little radioactive CO_2 was formed before the addition of succinate to the incubation flask. Furthermore, we have found that the formation of radioactive CO_2 paralleled the formation of δ-aminolevulinic acid (TABLE 9).

One cannot decide from these experiments if α-amino-β-ketoadipic acid is a free intermediate, for decarboxylation may occur by a concerted attack or while the α-amino-β-ketoadipic acid is still in linkage with the pyridoxal phosphate. The above-mentioned inhibition by δ-aminolevulinic acid would be consistent with the existence and formation of a pyridoxal phosphate derivative of δ-aminolevulinic acid.

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SOME REGULATORY FACTORS IN THE INTERCONVERSION OF GLUCOSE-6-PHOSPHATE AND RIBOSE-5-PHOSPHATE IN HUMAN ERYTHROCYTES

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Introduction

There is considerable evidence to indicate that the interconversion of glucose-6-phosphate (G-6-P) and ribose-5-phosphate (R-5-P) in animal tissues is involved in the synthesis of ribose mono- and polynucleotides and may also, under certain conditions, play a significant role as an energy-producing process.

The role of this interconversion in energy production is linked to its participation in the so-called direct oxidative pathway of glucose. In this metabolic pathway the conversion of ribulose-5-phosphate to fructose-6-phosphate and G-6-P represents a reversal of the two-step oxidation of G-6-P that produces ribulose-5-phosphate [some authors stress the fact that this oxidation of G-6-P produces reduced triphosphopyridine nucleotide (TPNH) that is necessary for numerous synthetic processes]. These synthetic processes, as well as the synthesis of ribose mono- and polynucleotides in general, will take place only at certain times and will not proceed continuously without interruption during the lifetime of a living cell. It seems reasonable, therefore, to assume that the direct oxidative pathway and the accompanying interconversion of ribose-5-P and G-6-P in living cells are released under certain conditions and suppressed under others. Variations in the activity of this enzyme system therefore should be subject to regulatory intracellular mechanisms, the nature of which is still completely obscure. A priori, it appears most probable that such regulations of enzymatic activities will be based on structural changes within the cell, but it is obvious that, in addition, certain much less complex factors that affect the kinetics of the respective enzymes, even *in vitro* and even in homogeneous solutions, may play a major role in this type of regulation. These factors now appear amenable to experimental analysis.

G-6-P can be regarded as a primary substrate for the direct oxidative pathway as well as the pentose phosphate cycle, as it is produced by the cell either directly from glucose or indirectly through the intermediary of glycogen. Although fructose-6-phosphate appears to be a substrate for transketolase and transaldolase, this ester is in equilibrium with G-6-P due to the activity of an isomerase present in high concentration in every living cell. The first factor that may determine the direct oxidation of G-6-P and its conversion to ribulose-phosphate and R-5-P may be the level of this ester in the cell. This level will be determined, among other factors, by the rate in which the ester is produced from glucose or glycogen by the activity of hexokinase and phosphorylase and by the rate by which it is resynthesized from its breakdown products. Therefore the concentration of G-6-P and fructose-6-phosphate will again be partly dependent upon the concentration of R-5-P, which in turn determines the concentration of ketopentose phosphate and other compounds produced by the transketolase and transaldolase systems; namely, erythrose-4- and glyceraldehyde-3-phosphate and its isomer dihydroxyacetone phosphate. The significance of the concentration of the intermediates not only depends on the reversibility of all the individual enzymes of the pentose phosphate cycle, but comes also from the characteristic feature of every cycle that the reaction product of any individual enzyme reaction serves as substrate in the subsequent reaction of the whole reaction chain.

In addition to the rate of formation of the substrates and intermediates and the rate of their breakdown, side reactions appear of importance as factors determining the intracellular concentration of these substances. As far as G-6-P is concerned, its oxidation to ribulose-5-phosphate and its conversion to R-5-P and ketopentose phosphates will compete with its phosphorylation to fructose-1,6-diphosphate by the phosphofructokinase, which represents the first step in the chain of glycolytic reactions. The complexity of the situation and of necessary regulatory processes is illustrated by the fact that the glycolytic system that competes for G-6-P with the direct oxidative pathway and the pentose phosphate cycle at the same time must produce an intermediate; namely, glyceraldehyde-3-phosphate which, in experiments with purified transketolase, appears necessary for the conversion of fructose-6-phosphate to xylulose-5-phosphate and, finally R-5-P.[1] As far as the intermediates in the pentose phosphate cycle are concerned, the side reactions that may be operative in the determination of their intracellular concentrations are partly of enzymatic and partly of nonenzymatic nature; they are due to the high reactivity of these substances. In the case of triose phosphates, erythrose-4-phosphate, and the two ketopentose phosphates this reactivity is linked to the fact that they are present in solution as aldehydes or straight chain sugars. The lability of the two ketopentose phosphates manifests itself in the rapid decay of these esters in simple salt solutions, particularly at alkaline *p*H.[2] The aldehydic nature of erythrose-4-phosphate is shown by its ability to condense with the dihydroxyacetone phosphate in the presence of muscle aldolase to sedoheptulose-1,7-diphosphate.[3] On the other hand, R-5-P can be assumed to be present predominantly in its fucanoid ring form. The fact, however, that it can function as acceptor for active glycolic aldehyde in the presence of transketolase and its substrates indicates that the transition of this ester from the ring form to the straight chain form can take place in solution at a significantly higher rate than is the case with other higher sugars or their esters.

The present report deals with two lines of experiments related to the problem of regulation of the rate of the interconversion of G-6-P and R-5-P. One was concerned with the significance of the concentration of glyceraldehyde-3-phosphate on the conversion of G-6-P and fructose-6-phosphate to R-5-P. The other deals with certain enzymatic and nonenzymatic reactions of glyceraldehyde-3-phosphate and R-5-P that could influence the intracellular concentration of these esters and thus indirectly the rate of the breakdown of G-6-P and fructose-6-phosphate.

Conversion of G-6-P to Sedoheptulose-7-Phosphate and R-5-P in the Absence of Triose Phosphate

It has been reported briefly[4] that, when G-6-P is added to a hemolyzate extensively dialyzed against $^1/_{80}$ M NaF, this ester is converted into a mixture of sedoheptulose phosphate, ribose- and ketopentose phosphate, triose- and erythrose-4-phosphate. After about $3^1/_2$ hours at 33°C., the reaction is completed and, at that point, about 80 per cent of the originally added hexose ester is in apparent equilibrium with the reaction product. This reaction is completely reversible, and R-5-P, added to the fresh hemolyzate, is partly converted to G-6-P and the other esters formed from glucose phosphate. However, if the hemolyzate, before addition of glucose phosphate, is incubated at 43°C. for 4 hours at *p*H 8.4 (Tris buffer) it loses its ability to break down the hexose ester, and the only well-defined reaction that takes place is the rapid conversion of G-6-P to fructose-6-phosphate, which usually is completed after about 15 min. This inactivated hemolyzate is able to convert R-5-P to equivalent amounts of sedoheptulose-7-phosphate, and triose phosphate, but not to hexose-6-phosphate.[5] It can be assumed that the incubation of the hemolyzate at 43°C. inactivates its transaldolase necessary for the reaction between sedoheptulose-7-phosphate and triose phosphate, leaving the transketolase system almost intact. If to the inactivated hemolyzate supplied with G-6-P we add fructose-1,6-diphosphate, which is rapidly converted by the muscle aldolase of the hemolyzate to triose phosphate, then sedoheptulose phosphate, pentose phosphate, and significant amounts of tetrose phosphate are rapidly formed, and the reaction comes to an equilibrium after about 1 hour at 33°C. If erythrose-4-phosphate is added to this mixture, G-6-P and fructose-6-phosphate are formed; however, addition of erythrose-4-phosphate alone without fructose-1,6-diphosphate does not induce any breakdown of glucose monophosphate. If erythrose-4-phosphate or a triose phosphate is added with G-6-P to the fresh hemolyzate, which contains the transaldolase, the formation of sedoheptulose phosphate is accelerated sharply. These observations suggest that the conversion of G-6-P and fructose-6-phosphate to other esters in the fresh hemolyzate is due to the simultaneous concerted activity of the transketolase and transaldolase systems in such a way that one molecule of fructose-6-phosphate transfers its two first carbons to glyceraldehyde-3-phosphate to form ribulose-5-phosphate, whereupon the remaining erythrose-4-phosphate reacts with another molecule of fructose-6-phosphate in the presence of transaldolase to yield sedoheptulose-7-phosphate and to restore the original concentration of triose phosphate. The accumulation of triose phosphate and erythrose-4-phosphate would then be due to the conversion of ribulose-5-phosphate by the transketolase to sedoheptulose phosphate and triose phosphate, and to the transketolase reaction between glyceraldehyde phosphate and G-6-P. This explanation, however, assumes the presence of an initial amount of triose phosphate sufficient to spark the transketolase reaction between fructose-6-phosphate and glyceraldehyde phosphate. Our experiments, however, show that the breakdown of G-6-P proceeds even in a most-exhaustively dialyzed hemolyzate that could not contain, therefore, even catalytic amounts of triose phosphate and fructose-1,6-diphosphate. It still seemed possible that very small amounts of either of these esters could have been present in the preparation of G-6-P that was added to the hemolyzate. This possibility was excluded in the following way. Human hemolyzates contain a very active glucose phosphate mutase that rapidly converts glucose-1-phosphate to G-6-P. Therefore, if glucose-1-phosphate, is added to the hemolyzate, it also is converted to the same mixture of sugar esters as G-6-P, the rate of the reaction being determined by the rate of the conversion of G-1-P. Glucose-1-phosphate is completely stable against alkali even at 100°C. Fructose-1,6-diphosphate and triose phosphates are rapidly destroyed under these conditions. In a series of experiments, therefore, M/125 solutions of K salts of glucose-1- and glucose-6-phosphates were heated for 30 min. in 0.2 N NaOH at 100° C., and the solutions were neutralized. One cc. of the heated solution was added to 3 cc. of the hemolyzate, and the sample was incubated for 3½ hours at 33° C. This was done at *p*H 8.4 and *p*H 7.2. As a control, corresponding solutions of glucose-1-P- and G-6-P that had not been heated with alkali were incubated under the same conditions. The formation of sedoheptulose-7-phosphate during the incubation period was then determined in all 4 samples by the cysteine H_2SO_4 reaction;[6] the results are presented in TABLE 1. As may be seen, the heating of glucose-1-phosphate did not affect in any way its breakdown to sedoheptulose phosphate. The preparation heated with alkali could not have contained any triose phosphate, but it was necessary to consider the possibility that extremely small amounts of fructose-1,6-diphosphate were still present. To estimate this amount, we carried out a control experiment in which the rate of degradation of fructose-1,6-diphosphate by 0.2 N NaOH at 100° C. was determined. The maximum possible content of fructose-1,6-diphosphate in the preparation of glucose-1-phosphate previously had been estimated by the cysteine-carbazol reaction to less than 1 per cent. From these data it could be calculated that the concentration of fructose-1,6-diphosphate in the hemolyzate with alkali-treated ester could not have been even 10^{-19} M. To determine whether a significant transketolation between fructose-6-phosphate and the triose ester could still occur at such concentration of glyceraldehyde-3-phosphate, the dependence of this reaction on the con-

TABLE 1

AMOUNTS OF SEDOHEPTULOSE IN HEMOLYZATE* FORMED FROM G-6-P AND G-1-P HEATED WITH NaOH AND UNHEATED

(Time 3½ hours; temperature 33° C.)

Substrate	Sedoheptulose at *p*H 8.4		Sedoheptulose at *p*H 7.2	
	Unheated	Heated	Unheated	Heated
Glucose-6-phosphate 2.3 μM/cc.........	105	0	162	0
Glucose-1-phosphate..	31	30	110	105

* In μg./cc.

centration of glyceraldehyde-3-phosphate in the range between 10^{-5} and 10^{-4} M was tested. The results indicated an approximately linear relation between the concentration of the triose ester and the conversion of the hexose ester to its reaction products. The turnover at the lower concentration of glyceraldehyde-3-phosphate at 10^{-5} M was less than one half the rate of the turnover of hexose-6-phosphate in the fresh hemolyzate to which no triose phosphate had been added. It is clear, therefore, that the amounts that could have still been present in the alkali-treated glucose-1-phosphate were completely negligible, even as catalysts, in the transketolase reaction. This work will be reported in detail later.

Reaction of Intermediates in the Pentose Phosphate Cycle Linked to Their Aldehydic Properties

Condensation of R-5-P with Dihydroxyacetone Phosphate in the Presence of Muscle Aldolase. Muscle aldolase condenses all aliphatic aldehydes and sugars with less than five carbons with dihydroxyacetone phosphate. Five- and six-carbon sugars, on the other hand, do not show any appreciable condensation in the presence of this enzyme. This is probably due to a very low concentration of the straight-chain form in solutions of this sugar. It seemed reasonable to expect that R-5-P may differ in this respect from other pentose hexoses and their esters, as its function as an acceptor in transketolase and transaldolase reactions indicated the availability of the straight-chain form in sufficient concentrations to yield significant turnover values in various enzyme reactions. It seems reasonable, therefore, to expect that this ester would condense with dihydroxyacetone phosphate in the presence of muscle aldolase to an octulose diphosphate. To test this assumption, a simple color reaction for the detection of octoses was developed.

Color reaction of octoses. This method is a modification of the color reaction of sugars with cysteine and sulfuric acid as previously described for hexose,[7] methyl-pentose, and heptose.[7] The procedure is as follows. To 1 cc. of the octose solution is added, under cooling in an ice water bath, 4.5 cc. of a mixture of 1 part of water and 6 parts of concentrated H_2SO_4. After 1 min. the mixture, in a test tube, is shaken in the ice water bath and then transferred first for a few minutes in tap water, then for 3 min. into a bath of vigorously boiling water, and then cooled again to room temperature. One tenth cc. of a 3 per cent solution of cysteine HCl is then added with shaking, and the sample is left overnight at room temperature. A blank containing water instead of octose solution is run simultaneously. Under these conditions gala-gala-octose and gluco-gala-octose yield a reddish-brown color with an absorption maximum between 465 and 470 mμ (FIGURE 1). Although they show the same maximum, the absorption curves for the two octoses so far tested are not completely identical.

Effect of water on the cysteine reaction of octose. After the development of the reddish-brown color, 2 cc. of water is added to the samples with cooling in tap water. The absorption maximum at 470 mμ disappears immediately, and a new purplish compound is formed that has a weak absorption maximum at 505 mμ.

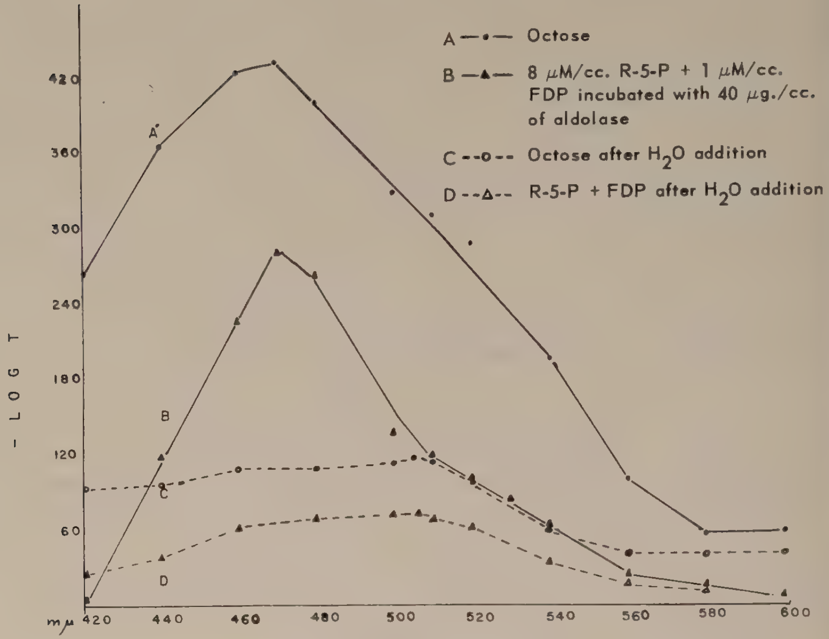

Figure 1

The cysteine H_2SO_4 reaction permits the detection of octoses, not only in pure solutions, but also in the presence of other sugars. Thus, by measuring the ratio of optical densities at 510 and 470 mμ, it is possible to find a mixture of octose in a solution containing heptoses and confirm the findings by showing an increase in the ratio D 505 mμ to D 470 mμ after the addition of water.

Formation from R-5-P of esters giving the reaction of octoses with cysteine and H_2SO_4. To demonstrate the combination of ribose-5-phosphate and dihydroxyacetone phosphate to a sugar ester that reacts in the cysteine reaction like an octose, the experiments were set up as follows. To 3 cc. of a 0.03 M Tris buffer of *p*H 7.4 were added 1 cc. of a 0.03 M solution of R-5-P, 1 cc. of a $1/100$ or $1/200$ M solution of fructose-1,6-diphosphate, and 0.1 cc. of the commercial suspension of twice recrystallized aldolase which was shown to contain a very active triose phosphate isomerase. This mixture was incubated at 33° C. for a period of 1 to 5 hours. As controls, we ran one sample of Tris buffer containing only R-5-P, another containing only Tris and fructose-1,6-diphosphate, and a third containing only the Tris buffer enzyme and 2 cc. of water. At the end of the incubation period the samples were deproteinized by adding 2.5 cc. of a 10 per cent trichloroacetic acid (TCA) solution left overnight at 4° C., and the precipitate was removed by centrifugation. The cysteine H_2SO_4 test for octose was then carried out on the experimental sample as well as on the controls, and a complete absorption spectrum was determined for all these samples. A difference-absorption curve was then determined by subtracting the optical density of the two controls from that of the experimental sample at every wave length. The same procedure was repeated after addition of 2 cc. of water to every sample to determine the shift in the absorption curve due to the presence of water. The difference-absorption curves before and after addition of water were then compared with the two absorption curves obtained with a standard of gluco-gala-octose; the 4 curves are presented in FIGURE 1. As can be seen, the difference-absorption curve of the experimental sample has a maximum at the same wave length as gluco-gala-octose, and this maximum shifts after addition of water in a way that corresponds to that of octose. The form of the difference-absorption curve is not completely identical, however, with that of gluco-gala-octose, which is not surprising as there are small differences also in the absorption curves of individual octoses.

Reactions of thiolic compounds with glyceraldehyde-3- and ribose-5-phosphate. Schubert[8, 9] showed in 1936 that various aldehydes in solution combine with cysteine, glutathione, and thiourea, forming two types of compounds, one in which only the thiolic group is linked to the carbon 1 of the aldehyde, and others in which a thioazolidine ring is formed by a combination of carbon 1 with the thiolic as well as with the amino group of cysteine. The stability of these compounds against weak alkali showed very great variations with the nature of the aldehyde and the nature of the thiolic compound. Schubert had already suggested that reactions of this type might play a role in the masking and unmasking of SH groups of proteins The aldehydes investigated in this type of reaction by Schubert and later by other authors were nonphysiological compounds; the kinetics of the reactions and their quantitative dependence on such factors as concentration and *p*H were not investigated in detail. Racker and Krimsky[10] briefly report that glyceraldehyde can combine with cysteine and glutathione, but do not state the exact experimental conditions. It appeared reasonable, however, to expect that the intermediates in the cellular metabolism that show aldehydic properties such as glyceraldehyde phosphate and erythrose phosphate might readily enter into such combinations with thiolic compounds that are abundantly present inside the living cells, and this type of reaction may significantly influence the course of metabolic processes. Experiments were therefore carried out in which the influence of cysteine, aminoethylmercaptan, and glutathione on metabolically active sugar esters and corresponding free sugars was examined. The experimental setup was the following. The solution of the sugar ester was added to the ninefold amount of a 0.1 M Tris buffer in the *p*H range between 7.35 and 9. Two samples were prepared. To one was added the thiolic compound the concentration of which varied from M/50 to M/125; to the other (control) sample an equal amount of water was added. The samples were incubated for 3½ hours in a water bath at 33° C. The reaction was interrupted by adding TCA to a concentration of 3 per cent, and the concentration of the sugar or sugar esters was determined simultaneously in the experimental sample and in the control, to which an equal amount of the thiolic compound was added after acidification with TCA. Free trioses were measured by the cysteine-carbazole reaction; R-5-P and ribose by Bial's orcinol reaction, according to Dische and Schwartz;[11] and hexoses and their esters by the reaction with cysteine and H_2SO_4. Fructose-6-phosphate and fructose-1,6-diphosphate were measured by a new modification of the cysteine-H_2SO_4 reaction, which will be described elsewhere, and which permits the determination of 10 μg. of a ketohexose in the presence of an excess of ketopentoses or ketoheptoses. The combination of the sugar with the thiolic compound was indicated by a decrease in the color reaction characteristic for the respective sugar, if the combination with the SH group was not hydrolyzed by acid. The stability of the compound against alkali was then determined by adding NaOH to a concentration of 0.4 M incubating the alkaline mixture for 25 min. at room temperature, neutralizing, and carrying out again the determination on the experimental sample and the control. The results of these experiments, except for those with glyceraldehyde-3-phosphate, are presented in TABLE 2. As may be seen, glyceraldehyde in a concentration of M/1000 disappears completely in the presence of M/50 cysteine. The disappearance could be determined by the cysteine-carbazole reaction because the compound with cysteine is stable with sulfuric acid at room temperature. Dihydroxyacetone does not react at all with thiolic groups. The aldehydic properties of R-5-P manifest themselves in its ability to combine with cysteine as well as with aminoethylmercaptan. The latter SH compound shows a much higher affinity to R-5-P than cysteine. However, the compound with mercaptan is much less stable in alkali, as 85 per cent of the pentose that has disappeared from solution is recovered from the compound with mercaptan after a 25-min. incubation in 0.4 N NaOH, while only about 10 per cent of the pentose is recovered under these conditions from the compound with cysteine. Glutathione did not have any effect on either glyceraldehyde or R-5-P. This indicates that the presence of the amino groups of cysteine or of the mercaptan is essential for the combination of these compounds with the sugars. Ribose itself did not show any significant reactions with cysteine.

The effect of thiolic compounds on glyceraldehyde-3-phosphate was measured indirectly in the following way. We added 0.5 cc. of a solution of fructose-1,6-diphosphate containing 5 μmole of this ester to 3 cc. of a solution prepared by adding 1 cc. of a suspension of commercial twice-recrystallized muscle aldolase* to 14 cc. of $\frac{1}{7}$ M Tris buffer of 7.4 or 8.4. To one of two such duplicate samples was added 0.5 cc. of M/6 solution of the thiolic compound, while the same amount of water was added to the other sample. Both samples were then incubated for 3½ hours at 33° C. and the reaction was interrupted by the addition of 2 cc. of 10 per cent TCA. The protein precipitate was centrifuged off, and fructose diphosphate and triose phosphate were determined in the supernatant of both samples. The preparation of muscle aldolase contained sufficient amounts of triose phosphate isomerase so that, if a certain amount of glyceraldehyde phosphate combined with thiolic groups, a certain amount of dihydroxyacetone phosphate was isomerized and a certain amount of fructose diphosphate was split into two molecules of triose phosphate. In our experiments the ratio between fructose diphosphate and triose phosphate, in the absence of thiolic compounds, was about 30:70. After incubation in

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* Obtained from Nutritional Biochemicals, Cleveland, Ohio.

TABLE 2

EFFECT OF THIOLIC COMPOUNDS ON VARIOUS SUGARS AND THEIR PHOSPHORIC ESTERS IN TRIS BUFFER

(Incubation 3½ hours at 33° C.)

Substrate	Concentration	Added SH compound	Before alkali treatment		Decrease due to SH compound in %	After alkali treatment		Recovery of bound R-5-P in %	pH
			Without SH compound	With SH compound		Without SH compound	With SH compound		
	μM/cc.								
(1) Ribose-5-phosphate	2.5	Cysteine M/50	2.33	0.58	75.0	2.18	0.75	9.6	7.35
(2) Ribose-5-phosphate	2.5	Cysteine M/50	2.21	0.50	77.0	2.08	0.73	12.5	8.40
(3) Ribose-5-phosphate	2.5	Cysteine M/125	2.21	1.51	32.0				8.40
(4) Ribose-5-phosphate	2.5	Cysteine M/125	2.21	1.57	29.0				9.0
(5) Ribose-5-phosphate	2.5	Amino ethylmercaptan M/125	2.21	0.56	74.0	2.08	1.98	93.5	8.4
(6) Ribose-5-phosphate	2.5	Glutathion	2.21	2.24	−1.5				8.4
(7) Ribose-5-phosphate	0.8	Cysteine M/125	0.80	0.75	6.0				9.0
(8) Glucose phosphate	63.0	Cysteine M/50	3.00	2.90	3.0				8.4
(9) Fructose-6-phosphate	1.5	Cysteine M/50	1.50	1.50	0				8.4
(10) Fructose-6-phosphate	1.5	Amino ethylmercaptan M/50	1.50	1.45	4.0				8.4

the presence of M/50 either of cysteine or of aminoethylmercaptan the concentration of fructose diphosphate went down to 0. This indicates that practically all of the triose phosphate must have combined with the thiolic compound. The direct determination of triose phosphate by the cysteine-carbazole reaction, however, showed the presence of as much triose phosphate in the samples with thiolic compounds as in those to which only water had been added. In experiments with glyceraldehyde itself, the triose bound by the thiolic compound was not detectable in the cysteine-carbazole test; therefore we must assume that the reaction products from glyceraldehyde phosphate and thiolic compounds are more labile to H_2SO_4 than the corresponding products formed from glyceraldehyde itself. Glutathione in concentration of 20 μM/cc. did not lead to any decrease of fructose diphosphate under the same conditions. It apparently does not combine significantly with glyceraldehyde phosphate, as in the case of glyceraldehyde and R-5-P.

The Effect of Thiolic Compounds on R-5-P and the Conversion of this Phosphate to Xyloheptulose-7-Phosphate and Triose Phosphate in Hemolyzates

When R-5-P is added to an inactivated hemolyzate it is partly converted within a few minutes to an equilibrium mixture of the two ketopentose-5-phosphates.[12] The pentose phosphates then are converted by a transketolase to a mixture of equivalent amounts of sedoheptulose-7-phosphate and triose phosphate. At concentrations of R-5-P below M/500, the rate of this conversion shows very nearly a linear dependence on the concentration of the pentose ester. To study the effect of cysteine, aminoethylmercaptan, and glutathione on this breakdown of R-5-P, 0.5 cc. of a solution containing 100 μmole of R-5-P was added to 3 cc. of a hemolyzate dialyzed against M/80 NaF. Next, 0.5 u. of a solution of a thiolic compound was added to one and 0.5 u. of water to the other of two such duplicate samples. Another set of duplicate samples to which an equivalent amount of water instead of R-5-P was added served as blanks. All samples were incubated for 3½ hours at 33° C., deproteinized with 2 cc. of 15 per cent TCA, and pentose, sedoheptulose, fructose-1,6-diphosphate, and triose phosphate were determined in the supernatants. In these determinations triose phosphate could not be determined directly by the cysteine-carbazole reaction because of the simultaneous presence in solution of three different types of keto sugars, all of which react more strongly than triose in this process. For this reason, the sum of fructose-1,6-diphosphate and triose phosphate was determined by a new method which will be described elsewhere and which is based on the conversion of all triose phosphate and fructose-1,6-diphosphate to erythrulose-1-phosphate by condensation of glyceraldehyde-3-phosphate with a large excess of formaldehyde in presence of the twice-recrystallized muscle aldolase preparation that contains an active triose phosphate isomerase. The determination of the tetrose ester was carried out by the characteristic cysteine-fructose-H_2SO_4 reaction. The other esters were determined by the methods mentioned above. The results of these determinations from two such experiments are shown in TABLE 3. As may be seen, in the presence of cysteine a significantly greater amount of ribose disappeared from the hemolyzate than in the absence of this compound. Glutathione again had no such effect. At the same time, the amount of sedoheptulose formed was increased in one experiment, very significantly, by as much as 22 per cent. In this experiment the amount of triose phosphate formed without cysteine in an amount equivalent to that of sedoheptulose was significantly smaller in the cysteine sample. Fructose diphosphate, on the other hand, decreased by more than 80 per cent under the influence of cysteine. This was much more than could be explained by the shift in the

TABLE 3

EFFECT OF CYSTEINE (M/50) ON THE BREAKDOWN OF R-5-P IN DIALYZED AGED HEMOLYZATES

(Incubation 3½ hours at 33° C., *p*H 8.4, M/60 NaF)

Experiment No.	Incubation	Decrease in total pentose in percentage of initial value		Recovery in alkali of pentose bound by cysteine in %	Sedoheptulose formed	FDP + triose P formed as TrP	FDP formed
		Before alkali treatment	After alkali treatment				
					μM/cc.	*μM/TrP/cc.*	*μM/cc.*
I	a. R-5-P 10 μM/cc. without cysteine	53.0	50.5		3.06	2.98	0.43
	b. with cysteine	75.5	33.0	91	3.73	2.53	0.05
II	a. R-5-P 5 μM/cc. without cysteine	78.0	77.5		1.65		
	b. with cysteine	95.0	59.0	106	1.68		

equilibrium with muscle aldolase due to the decrease of triose phosphate. As the sum of fructose diphosphate and triose phosphate in this case was determined by condensing glyceraldehyde phosphate in the presence of muscle aldolase and isomerase with formaldehyde to erythrose-1-phosphate, this discrepancy can be explained by the formation of a dissociable complex between cysteine and glyceraldehyde phosphate. As soon as the ester is removed by condensation with formaldehyde, the complex partly dissociates, and freed glyceraldehyde phosphate can again combine with formaldehyde until a new equilibrium is reached. The decrease in R-5-P in these two experiments greatly exceeds that calculated from the increase in sedoheptulose-phosphate. When the TCA extracts were treated with alkali, all pentose that disappeared without breaking down to sedoheptulose and triose was recovered. This result is completely at variance with the results of the experiments on the effect of cysteine on R-5-P in pure solution. In the latter experiments only about 10 per cent of the ribose that reacted with cysteine could be recovered by alkali hydrolysis. Aminoethylmercaptan, on the other hand, in pure solution yielded a compound from which almost all of the pentose could be recovered. In hemolyzates, therefore, cysteine appears to react in the same way as aminoethylmercaptan in pure solution.

Comments

Our experiments indicate that the conversion of G-6-P to R-5-P can be independent to a high degree of the activity of the glycolytic system. This independence is based on two facts:

First, the ability of G-6-P to produce for itself a sufficiently active acceptor in the transketolase reaction of fructose-6-phosphate that is not identical with glyceraldehyde-3-phosphate. The nature of this acceptor, however, remains hypothetical at this time. This conclusion can be drawn from the fact that the rate of the reaction between fructose-6-phosphate and glyceraldehyde-3-phosphate in the presence of transketolase is too small at a concentration of glyceraldehyde phosphate below 10^{-4} to be able to explain the rate of the conversion of G-6-P to R-5-P in the hemolysate to which no glyceraldehyde phosphate was added. It does not appear, therefore, necessary that fructose-1,6-diphosphate and triose phosphate be formed by the activity of phospho-fructo-kinase and muscle aldolase.

Second, glyceraldehyde phosphate can play a role as an acceptor for the carbon 1 and 2 of fructose-6-phosphate, so that the addition of these esters to a hemolyzate containing G-6-P will increase the rate of formation of R-5-P from the hexose ester. This situation amounts to a feedback mechanism that provides a certain degree of stability as far as the rate of conversion of G-6-P to R-5-P is concerned. An increase in the phosphorylation of fructose-6-phosphate will tend to decrease the concentration of the hexose ester and, therefore, the rate of its conversion, but at the same time will tend to increase the concentration of glyceraldehyde phosphate formed from fructose-1,6-diphosphate, which will tend to accelerate this same conversion.

Our observations of the aldehydic properties of R-5-P and its ability to react with thiolic groups in a similar way as glyceraldehyde-3-phosphate and, most probably, erythrose-4-phosphate suggest the possibility of another regulatory mechanism in the living cells. This would affect the formation of R-5-P either by the transketolase-transaldolase system or the whole shunt mechanism. Although glutathione was not active in our experiment as far as binding of the aldehyde intermediates is concerned, and free cysteine cannot be expected to be present in sufficient concentration to affect significantly the concentration of the intermediates, active sulfhydryl groups of cellular proteins could be effective in this respect. For instance, cysteine end groups with free amino groups could bind glyceraldehyde, erythrose, and R-5-P more efficiently than cysteine itself, as is the case with aminoethylmercaptan. On the other hand, the fact that cysteine in hemolyzates apparently binds the intermediate esters to form compounds more labile to alkali than cysteine itself indicates the ability of hemoglobin to modify the reactivity of cysteine, perhaps by tying up the carboxyl group of cysteine by hydrogen bondings or linking to free amino acid groups of the protein. It is possible, therefore, that specific configurations of protein molecules that, due to specific hydrogen bondings, result in a close proximity of the free amino group of lysine to the thiolic group of cysteine, could result in a highly efficient binding of the intermediary esters. However, further investigations are necessary to determine the nature of the ester that can be recovered from the combination with cysteine in hemolyzates.

Acknowledgment

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SOME ASPECTS OF THE METABOLISM OF RED BLOOD CELLS FROM PATIENTS WITH HEMOLYTIC ANEMIAS*

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During recent years we have been engaged in studying abnormal red blood cells (RBC) from patients with various types of hemolytic anemias. This investigation was directed toward the delineation of some of the metabolic parameters, in particular those pertaining to carbohydrate metabolism. From the inception of this investigation we hoped not only that knowledge concerning the metabolic behavior of abnormal RBC would contribute to a better understanding of the underlying mechanisms for the increased rate of destruction of circulating RBC encountered in patients with hemolytic anemia, but also that such knowledge would also shed light upon the normally occurring rate of destruction of senescent circulating RBC. Although no precise answer can be given to the questions posed as a result of the preceding consideration, the experimental approach discussed here and some of the results presented will serve as the basis for assessing the present status of these studies, as well as their presumed future direction.

The premise that the cellular integrity of the mature human RBC is largely dependent upon the functional adequacy of glycolysis is fundamental to the experimental approach adopted in this work. Such a premise appears reasonable in view of the fact that in the human mature RBC anaerobic glycolysis constitutes one of the most important metabolic activities to which these cells have developed a unique degree of adaptation, considering their lack of a nucleus and of extensive and intensive biosynthetic activity. It is therefore considered likely that alterations in one of the constituent reactions of the multienzyme system referred to as "anaerobic glycolysis" could lead to the impairment of the viability of RBC and thus produce hemolysis. Such considerations are of particular pertinence when one deals with hemolytic anemias, in which the hemolytic tendency is a genetically determined characteristic; by analogy, it may be speculated in such circumstances that the gene responsible for production of the hemolytic tendency regulates the activity of a specific enzyme participating in one of the glycolytic reactions.

It is probably a universal truth that in tissues under *in vivo* conditions the enzyme concentration exceeds that of the substrate; that is, that the enzyme is not completely saturated with substrate. It thus appears possible that a lowered enzyme concentration present in an abnormal cell would not be detectable until such enzyme concentration becomes limiting in the test system used. In an attempt to approximate more clearly such a limiting level of the enzyme concentration and thus to facilitate the detection of metabolic changes in the abnormal RBC, a variety of inhibitors was employed in these experiments.

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The metabolic activity of the mature circulating RBC may be expected to be influenced decisively by the nature and concentration of the substances traversing the semipermeable membrane that envelops the cell. Furthermore, the course and rate of entry of potential substrates into the cell could be directed effectively by the enzymatically catalyzed reaction occurring at the site of entry into the cell, namely, at or near its surface. Evidence for glycolytic activity at the surface of human RBC and for the possible existence of a metabolically autonomous compartment within the surface of the RBC has been presented by one of us in collaboration with Prankerd[1] several years ago. These earlier studies have provided a conceptual basis for the experiments discussed here. Thus, the rate of entry of orthophosphate-P^{32} was employed as a means of studying the over-all metabolic activity of the RBC, as well as of the metabolic events involved in the movement of inorganic phosphate from the outside of the cell to its interior. On the basis of the course of P^{32}-labeling of organic esters of phosphoric acid inside the RBC and because of other criteria, it has been assumed[1] that inorganic phosphate undergoes esterification in the course of entry into the cell as a result of reacting with glyceraldehyde-3-phosphate, a reaction catalyzed by the enzyme triosephosphate dehydrogenase.[1] The rate of entry by orthophosphate-P^{32} was measured by determining the rate of disappearance of radioactivity due to P^{32} from the plasma during *in vitro* incubation of whole blood at 37° C. for several hours. During these incubations the *p*H of the system was maintained at a constant level by means of bicarbonate buffer. The chemical quantity of orthophosphate-P^{32} added to the system was negligible with respect to the total inorganic phosphate concentration; consequently, no change in the chemical concentration of intracellular and extracellular phosphate occurred during the period of incubation.

Since orthophosphate is known to leave the RBC[2] by mechanisms as yet undetermined, RBC obtained from patients with hemolytic disease have been studied in order to evaluate this aspect of the metabolic activity of abnormal RBC. To this end, whole blood was incubated for 1 hour with orthophosphate-P^{32} under the conditions described above. The RBC thus labeled were sedimented by centrifugation, washed, resuspended, and finally reincubated in compatible plasma for several hours, during which period the rate of appearance of radioactivity in the plasma was determined.

Some results obtained from studies of the rate of disappearance of orthophosphate-P^{32} from the plasma of blood samples obtained from patients with either hereditary spherocytosis or with paroxysmal nocturnal hemoglobinuria, two different types of hemolytic anemia investigated, are shown in TABLE 1, whereas results obtained from studies of the rate of appearance of orthophosphate-P^{32} from labeled cells in the plasma are shown in TABLE 2, again juxtaposing the 2 types of hemolytic anemia mentioned previously.

The data shown in TABLE 1 reveal several differentiating features of the two types of hemolytic anemia studied:

(1) The rate of entry into the RBC of orthophosphate-P^{32} is within normal limits in the case of RBC from patients with hereditary spherocytosis and in the absence of NaF, whereas the rate of entry of the labeled phosphate is

TABLE 1

RATE OF DISAPPEARANCE OF P^{32} ACTIVITY FROM PLASMA

Source of red blood cells	Additions to incubation mixture	$t_{0.5}$* hr.
Hematologically normal donors	None	1.9
	1×10^{-3} M NaF	1.9
Patients with hereditary spherocytosis	None	1.9
	1×10^{-3} M NaF	4.0
Patients with paroxysmal nocturnal hemoglobinuria	None	3.9
	5×10^{-3} M NaF	3.4

* The symbol $t_{0.5}$ = time in hours required for the disappearance of one half of the P^{32} activity added to whole blood at the start of the incubation period. The values for $t_{0.5}$ represent the means obtained from a study of 5 hematologically normal donors, 6 patients with hereditary spherocytosis (3 of whom had been splenectomized), and 2 patients with paroxysmal nocturnal hemoglobinuria, one of whom was studied repeatedly. All values are significant in the range of $p < 0.002$.

TABLE 2

RATE OF APPEARANCE OF P^{32} ACTIVITY IN THE PLASMA

Source of P^{32}-labeled red blood cells	Additions to the incubation mixture	k_r,* hr.$^{-1}$
Hematologically normal donors	None	3.14
	5×10^{-3} M NaF	3.68
Patients with hereditary spherocytosis	None	2.38
	5×10^{-3} M NaF	1.67
Patients with paroxysmal nocturnal hemoglobinuria	None	0.49
	5×10^{-3} M	0.61

* The symbol k_r = first-order reaction rate constant calculated for the initial reaction velocity from a plot of the P^{32} activity in the plasma versus time in hours. The values for k_r represent the means obtained from the study of 3 hematologically normal donors, 3 patients with hereditary spherocytosis, all of whom had been splenectomized, and 2 patients with paroxysmal nocturnal hemoglobinuria. All values are significant in the range of $p < 0.002$.

markedly decreased in RBC from patients with paroxysmal nocturnal hemoglobinuria.

(2) Although the addition of 5×10^{-3} M NaF normally does not affect the rate of disappearance of orthophosphate-P^{32} from the plasma, the concentration of NaF has a pronounced inhibitory effect on the same process in RBC of patients with hereditary spherocytosis.

(3) In contrast with RBC from patients with hereditary spherocytosis, 5×10^{-3} M NaF causes a moderate increase in the rate of disappearance of P^{32} activity from plasma. However, the addition of NaF does not effect a return to a normal rate of disappearance.

In contrasting the biochemical features of the two types of hemolytic anemias under discussion, it has been found that other metabolic inhibitors, such as iodoacetic acid, have no differentiable effect on the abnormal RBC. It is of interest, however, to note that the addition of purine nucleosides (adenosine, for example), will bring about a reversal of the inhibitory effects of NaF, as well as of iodoacetic acid, even when these inhibitors are present in the same incubation mixture, as has been observed[3] in the case of RBC from hereditary spherocytosis.

In view of the well-established fact that fluoride ion inhibits glycolysis at the level of enolase and in view of Prankerd's observation (personal communication) that phosphatases, including adenosine triphosphatase, are present in normal concentrations in RBC from patients with hereditary spherocytosis, the inhibitory effect of fluoride ion on the transport of orthophosphate-P^{32} across the cell boundary is regarded as a reflection of a deficiency with respect to enolase in RBC from the patients previously mentioned. Insofar as the transport of phosphate into the RBC is concerned, this deficiency may assume critical proportions only in relation to that fraction of total enolase of the RBC that is present at or near the cell surface; the effect of such a postulated enzyme deficiency, however, may be more far reaching and may involve a variety of processes that require a functionally adequate glycolytic system at the cell surface.

The accelerating action of NaF on the rate of P^{32} transport in RBC from patients with paroxysmal nocturnal hemoglobinuria is considered to constitute evidence for the existence of a metabolic imbalance, perhaps with respect to the ratio of adenosine diphosphate to adenosine triphosphate, which could lead to a temporary acceleration in the rate of formation of triosephosphates. This unusual effect of fluoride ion may be viewed as being superimposed upon an intrinsic defect that is characteristic of this type of abnormal RBC in which the concentration of adenosine diphosphate may be inadequate for the maintenance of a normal rate of regeneration of adenosine triphosphate.

The data shown in TABLE 2 must be interpreted in the light of the labeling pattern of the intracellular phosphorus-containing compounds, since the RBC are incubated for 1 hour prior to being transferred to fresh autogenous plasma. Data concerning the labeling pattern of normal circulating human RBC and RBC from patients with hereditary spherocytosis are available,[1, 4] whereas data for RBC from patients with paroxysmal nocturnal hemoglobinuria, although available, are as yet unpublished. In the former type of hemolytic anemia the intracellular labeling pattern deviates from the normal pattern in that the specific P^{32} activity of inorganic phosphate rises much more rapidly and to values exceeding several times that normally found; in relation to other phosphorus-containing compounds in the cell, the P^{32} activity is considerably higher than the P^{32} activity of the adenosine polyphosphates and 2,3-diphosphoglyceric acid that normally incorporate the greatest quantity of P^{32}. In the case of RBC from patients with paroxysmal nocturnal hemoglobinuria, the intracellular P^{32} labeling has revealed a lower rate of turnover of phosphorus in 2,3-diphosphoglyceric acid, as well as a high level of isotope concentration in the stromal fraction prepared as described elsewhere[1] than is true of normal RBC.

It is apparent from TABLE 2 that, despite the great facility and rapidity with which orthophosphate-P^{32} enters the cell,[4] RBC from patients with hereditary spherocytosis lose P^{32} activity at the rate of about 75 per cent of the normal rate. This rate is further retarded by the addition of fluoride, which causes a decrease in the rate of appearance of P^{32} in the plasma by approximately 45 per cent, although the normal rate in the presence of fluoride is slightly elevated as compared with the rate in the absence of fluoride. In the case of paroxysmal nocturnal hemoglobinuria, the rate of appearance of P^{32} activity in the plasma is almost the same with and without addition of NaF, but represents only 15 per cent of the rate normally found. These findings suggest that RBC from patients with paroxysmal nocturnal hemoglobinuria exhibit a decreased rate of efflux of orthophosphate-P^{32} in either direction irrespective of the addition of fluoride. In this respect, RBC from patients with hereditary spherocytosis also behave differently from the afore-mentioned cells, since the decrease in the rate of P^{32} efflux is not as great, and since there is a detectable additional decrease in this rate upon the addition of NaF.

If the rate of transport of orthophosphate-P^{32} in and out of the RBC can be regarded as an index of the metabolic activity of the surface of the cell, then it may be postulated that the metabolic abnormalities described in this communication are related directly to changes in the structure of the cell surface and to the localization of enzymic units therein. It is thus understandable why cells altered in such a way would be subject to more rapid destruction and would lose their cellular integrity more rapidly than would normal RBC.

It should be pointed out here that the cell population present in the samples of whole blood used for incubation is probably a heterogeneous one, and that the variation between individual cells is probably considerable as far as altered metabolic activity and hemolytic tendency are concerned. It remains unknown at this time whether certain metabolic parameters are altered in all of the abnormal cells. In spite of the possible heterogeneity of the population of RBC, the over-all changes in the parameters investigated here must have been of sufficient magnitude to permit detection.

Although it is possible, in a general way, to relate an increased hemolytic tendency to metabolic changes, the details of the varied mechanisms causing the impairment of viability of abnormal RBC remain to be elucidated. The findings presented here suggest that loss of cellular integrity in hemolytic anemias may occur as a result of different metabolic changes, as exemplified by the comparison of hereditary spherocytosis and paroxysmal nocturnal hemoglobinuria, and that a specific metabolic abnormality is not common to both types of hemolytic disease.

Summary

(1) Two types of hemolytic anemia have been studied with respect to transport of orthophosphate-P^{32} into and out of the circulating red blood cell. (2) The results of such experiments have been discussed in relation to the impairment of cellular integrity of RBC from patients with hereditary sphero-

cytosis or paroxysmal nocturnal hemoglobinuria, and have been compared with results obtained from RBC of hematologically normal donors. (3) The data presented suggest that different types of hemolytic anemias are associated with different metabolic changes, and that in hereditary spherocytosis and paroxysmal nocturnal hemoglobinuria, such changes occurring at the cell surface may play a major role in the maintenance of cellular integrity.

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ADENOSINE TRIPHOSPHATE METABOLISM IN THE RABBIT ERYTHROCYTE *IN VIVO* AND *IN VITRO**

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The mechanism associated with the control of the life span of the mammalian erythrocyte *in vivo* presents a problem of major biological interest. Although little is known concerning the sequence of events leading to the destruction of the erythrocyte at the end of a predictable period of time, it is probable that the initiating mechanism resides within the cell itself.[2] A study of the erythrocyte with respect to certain constituents the concentrations of which may change during the aging process *in vivo* may provide a clue to the process by which the destruction of the erythrocyte is initiated.

The energy metabolism of the erythrocyte is primarily dependent upon the utilization of glucose that enters the cell from the plasma by way of an active transport mechanism and is converted to glucose-6-phosphate. This reaction is catalyzed by hexokinase and requires adenosine triphosphate (ATP) as a specific coenzyme. The subsequent metabolism of the glucose-6-phosphate provides a major source of energy for the maintenance of the cell.

The role of adenosine triphosphate in biological processes is unique. In addition to serving as a specific coenzyme for many metabolic reactions, it is also a precursor of a number of coenzymes and a mediator of energy transfer. During the aging of the erythrocyte *in vitro* it is well known that the level of ATP declines progressively with a concomitant loss of viability of the stored erythrocyte.[3] A relationship of this loss of viability to the ATP level of the erythrocyte is implied by the finding that storage in media that maintain the ATP content for longer periods of time also results in a prolongation of the viability of the erythrocyte.[4] Although ATP is multifunctional, the maintenance of the erythrocyte is probably related to the optimal functioning of the hexokinase reaction.

The rapid turnover of the high-energy terminal phosphate groups of ATP in the erythrocyte has been studied.[5] However, a limiting factor for ATP formation may be a diminished content of receptor molecules, that is, adenosine monophosphate (AMP) or adenosine diphosphate (ADP), for the attachment of one or more terminal phosphate groups. This, in turn, will relate to the capacity of the circulating erythrocyte for replacement of losses of adenine nucleotides that may occur within the individual erythrocyte during the aging process. Jørgensen has reported that such losses occur during aging *in vitro* with the accumulation of free purines.[6]

An investigation of the biosynthesis and metabolism of ATP in the mature mammalian erythrocyte *in vivo* was undertaken in order to determine whether the ATP of the erythrocyte in the peripheral circulation resembles cholesterol[7] or glutathione,[8] both of which undergo metabolic turnover, or whether each newly formed erythrocyte is released into the circulation with a fixed content of ATP, which then must suffice for the life of the cell. In the absence of a biochemical mechanism in the erythrocyte for the formation of adenine nucleotides, losses of ATP associated with aging could result in an eventual depletion of this compound from the cell.

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The question was investigated in a group of adult New Zealand white rabbits by the subcutaneous administration of glycine-2-C^{14}, a known precursor of the heme moiety of hemoglobin[9] and of the adenine portion of ATP.[10] At various time intervals, the animals were exsanguinated and, from the washed erythrocytes, hemoglobin and ATP were isolated. Hemin was prepared from the hemoglobin and was recrystallized to constant radioactivity by standard procedures. The ATP was obtained by ion exchange chromatography[11] and was then hydrolyzed. The specific activity of the adenine was determined after elution from an ion exchange column. Purity and constancy of specific activity were established by paper chromatography in several solvent systems.

The results are shown in FIGURE 1, where specific activity is plotted against time following glycine-2-C^{14} administration. The curve obtained from the heme data is similar to that previously reported by Neuberger and Niven[12] for the rabbit erythrocyte, the life span of which is about 65 days. The absence of a flat plateau has been ascribed by these authors to a small degree of random destruction of the erythrocytes of some rabbits.

In contrast to the relative metabolic stability of the heme, the metabolic behavior of the adenine of ATP is clearly different, since the specific activity of the adenine declined exponentially after several days and reached a very low value after about 55 days. The half life was calculated to be about 13 days.

In order to explain the decline in the specific activity of the adenine of ATP, several possibilities which may not be mutually exclusive, must be considered.

The possible interpretations associated with a renewal mechanism within the individual erythrocyte during the aging process include: (1) the exchange of erythrocyte ATP with that of plasma, (2) *de novo* synthesis of the adenine of ATP from simple precursors available to the circulating erythrocyte, (3) the exchange of the intact adenine moiety alone, (4) the exchange of the adenosine portion, and (5) the utilization of an intermediate compound having a partially formed purine ring structure, supplied to the erythrocyte and subsequently converted to ATP by the cell.

The first possibility, namely, the exchange of erythrocyte ATP with ATP of plasma, may be dismissed because of the absence of detectable ATP in rabbit plasma and of the impermeability of the erythrocyte to this compound.

The possibility of *de novo* synthesis of the adenine of ATP has been investigated *in vitro* with the use of glycine-2-C^{14} alone and supplemented with the small molecule purine precursors. In addition to glycine, these include sodium formate, sodium carbonate, L-glutamine, and L-aspartic acid. All *in vitro* experiments included glucose supplementation. The results of several of these experiments are summarized in TABLE 1. Incubation of freshly drawn washed

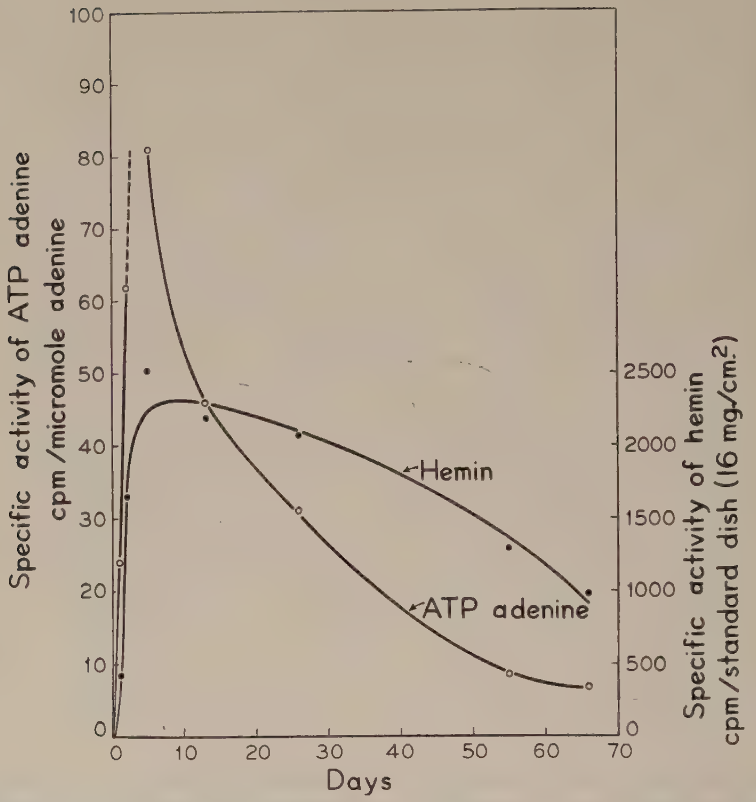

FIGURE 1. Incorporation of glycine-2-C^{14} into the adenine moiety of adenosine triphosphate and into the hemin of hemoglobin.

erythrocytes with glycine-2-C^{14} in plasma at 37° C. for 3 or 6 hours resulted in no labeling of the adenine, nor was the adenine labeled when the glycine-2-C^{14} was supplemented with all of the small-molecule precursors. Although the erythrocyte is impermeable to phosphorylated compounds, in several experiments both ATP and guanosine triphosphate (GTP) were added, since a number of reactions that lead to purine biosynthesis require one of these coenzymes, and it appeared possible that some of the purine biosynthetic reactions might occur at the erythrocyte membrane. As a result of the recent finding of Allfrey and Mirsky[13] concerning the polynucleotide requirement for ATP formation by phosphorylation reactions in calf thymus nuclei, rabbit sodium nucleate was also added. The mixed nucleic acids were obtained by hot 10 per cent sodium chloride extraction of dry tissue (liver, kidney, and intestine) for a related study that will be reported elsewhere. No isotopic labeling of the ATP was found with this additional supplementation.

Isotonic saline was used as the suspending medium in one of the experiments, in order to eliminate the possibility of inhibition of ATP synthesis, *in vitro*, by plasma, but again no labeling resulted.

TABLE 1

STUDY OF *DE NOVO* ATP BIOSYNTHESIS IN RABBIT ERYTHROCYTE *IN VITRO*
(Incubation with glycine-2-C^{14} at 37° C.)

Incubation medium	Glycine-2-C^{14}		Supplement	Incubation time	ATP-adenine
	μc	*mmoles*		*hours*	*cpm/μmole*
Plasma	52	0.05	glucose (0.46 mmoles)	6	<1
Plasma	40	0.16	glucose* purine precursors†	3	<1
Plasma	40	0.16	glucose* purine precursors† ATP (16.0 μmoles) GTP (11.0 μmoles)	3	1
Plasma	40	0.16	glucose* purine precursors† ATP (16.0 μmoles) GTP (5.0 μmoles) sodium nucleate (5.0 mg.)	3	<1
Isotonic saline	40	0.16	glucose* purine precursors† ATP (16.0 μmoles) GTP (5.0 μmoles) sodium nucleate (5.0 mg.)	3	1

* Glucose: concentration, 0.2 mmole/hr./100 ml. erythrocytes; total mmoles, 0.24.

† Purine precursors: L-glutamine, L-aspartic acid, and sodium formate; concentration, 0.2 mmole/hr./100 ml. erythrocytes; total mmoles, 0.24; also, sodium carbonate; concentration, 0.1 mmole/hr./100 ml. erythrocytes; total mmoles, 0.12.

In other experiments, the use of sodium formate-C^{14}, either alone or supplemented with the small-molecule purine precursors, failed to result in labeling of the adenine portion of ATP.

Although these experiments *in vitro* do not preclude the possibility of *de novo* purine synthesis *in vivo*, they do suggest that the intact sequence for purine biosynthesis *de novo* may indeed be lacking in the mature mammalian erythrocyte. It is known that nucleic acid synthesis does not occur in this cell. It is of interest to note that the mature mammalian erythrocyte also lacks the intact Krebs tricarboxylic acid cycle, the cytochrome system, and the ability to synthesize proteins, including hemoglobin.

The possibility of turnover of the adenine moiety of ATP is currently under investigation. Preliminary data indicate that incubation of rabbit erythrocytes in plasma at 37° C. for 3 hours with adenine-8-C^{14} results in considerable labeling of the purine portion of ATP. Preiss and Handler[14] have found that an acetone powder extract of human erythrocytes effected the synthesis of adenylic acid from added adenine in the presence of 5-phosphoribosyl 1-pyrophosphate (PRPP). Although the intact erythrocyte appears to possess the ability to utilize preformed adenine for ATP synthesis, this pathway may not account for any significant nucleotide synthesis in the circulating erythrocyte in view of the insignificant purine level of normal plasma.

The possibility of adenosine utilization is also under study, and it appears that adenosine-8-C^{14}, when incubated under conditions similar to those described for adenine-8-C^{14}, will also give rise to ATP with considerable labeling

TABLE 2

POSSIBLE METABOLIC PATHWAYS LEADING FROM ADENOSINE TO ATP

$$\begin{tikzcd}
\text{Adenosine} \arrow[r, leftrightarrow] \arrow[d, leftrightarrow] & \text{Inosine} \arrow[r, leftrightarrow] & \text{Hypoxanthine} \arrow[dl, leftrightarrow] \\
\text{Adenylic Acid} \arrow[r, leftrightarrow] \arrow[d, leftrightarrow] & \text{Inosinic Acid} & \\
\text{ATP} & &
\end{tikzcd}$$

in the adenine portion. The possible mechanisms summarized in TABLE 2 are being investigated further. Rabbit erythrocytes are known to contain both adenosine deaminase[15] and purine nucleoside phosphorylase[16] that could effect a deamination of adenosine and a conversion of the resultant inosine to hypoxanthine and ribose-1-phosphate. A more definitive experiment using adenosine labeled in both purine and ribose moieties should indicate whether the adenosine per se is utilized for this conversion to ATP, or whether only the purine moiety is used. The significance of this adenosine utilization, however, may be questioned in view of the apparent absence of purine nucleosides in normal plasma.

The final interpretation compatible with a turnover mechanism is based on the possibility that, although the over-all purine biosynthetic sequence may be lacking, the erythrocyte may possess the enzymatic apparatus for the conversion to a purine nucleotide of an intermediate on the pathway of purine biosynthesis, with subsequent conversion of the purine nucleotide to ATP. To test this hypothesis, a compound related to the most immediate purine precursor was studied. The work of Greenberg[17] and of Flaks *et al.*[18] has implicated 5-amino-4-imidazole carboxamide ribotide as an immediate purine precursor. The aglycone of this compound requires only a single carbon atom to become a purine, and this carbon atom of the purines is known to arise from a "one-carbon" donor such as formate.

Because of the reported impermeability of the erythrocyte to phosphorylated compounds, 5-amino-4-imidazole carboxamide riboside (FIGURE 2) was studied instead of the corresponding ribotide. Sodium formate-C^{14}, supplemented

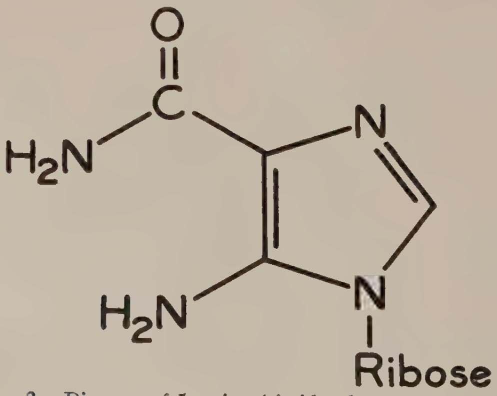

FIGURE 2. Diagram of 5-amino-4-imidazole carboxamide riboside.

TABLE 3

STUDY OF ATP-ADENINE BIOSYNTHESIS IN RABBIT ERYTHROCYTE *IN VITRO* FROM 5-AMINO-4-IMIDAZOLE CARBOXAMIDE RIBOSIDE AND SODIUM FORMATE-C^{14}

Rabbit erythrocytes (33 ml.) incubated in plasma (33 ml.) for 3 hours at 37° C.

Incubation suspension contains:	Concentration	ATP-adenine
	mmole/hr./100 ml. cells	*cpm/μmole*
(1) Sodium formate-C^{14} (40 μc),	0.2	4
glucose, L-aspartic acid, L-glutamine,	0.2	
glycine, sodium carbonate, inosine	0.1	
(2) Sodium formate-C^{14} (40 μc),	0.1	6500
L-aspartic acid, 5-amino-4-imidazole carboxamide riboside,	0.1	
glucose	0.2	

with the small molecule purine precursors, as well as with glucose and inosine, was incubated with washed rabbit erythrocytes in plasma at 37° C. for 3 hours, and the specific activity of the ATP adenine was determined. Inosine was included in order to provide potential ribose-5-phosphate, by way of a mutase reaction, from the ribose-1-phosphate formed by the action of purine nucleoside phosphorylase on the inosine. In a similar manner, sodium formate-C^{14} was supplemented with 5-amino-4-imidazole carboxamide riboside, L-aspartic acid, and glucose, and the mixture was incubated. Aspartic acid was added to this incubation mixture since it is the specific precursor of the 6-amino group of adenine. Subsequent experiments have indicated that this addition was unnecessary. Any requirement for this amino acid is apparently satisfied by the aspartic acid level in the incubation suspension.

A comparison of the specific activities of the adenine obtained from the ATP preparations is shown in TABLE 3. Although no incorporation of formate-C^{14} was observed in the presence of the small molecule purine precursors, a considerable uptake into the adenine moiety of ATP occurred in the presence of 5-amino-4-imidazole carboxamide riboside. The capacity of the rabbit erythrocyte to effect the final steps of *de novo* purine biosynthesis has thus been demonstrated, in spite of an apparent inability to effect over-all *de novo* synthesis from the readily available simple precursors of the purine ring.

The reaction sequence probably involves a phosphorylation of the 5-amino-4-imidazole carboxamide riboside to the corresponding ribotide by a kinase similar to that recently isolated by Flaks *et al.* from yeast and pigeon liver.[19] This is followed by formylation and ring closure to form inosinic acid, labeled in carbon 2 of the purine. Presumably, inosinic acid is then converted to adenylic acid by the mechanism described by Lieberman,[20] with subsequent phosphorylation to ATP.

The possible utilization of 5-amino-4-imidazole carboxamide riboside by phosphorolytic cleavage to form the 4-amino-5-imidazole carboxamide appears to be relatively unimportant, since it was found that only a slight incorporation of formate-C^{14} into ATP adenine resulted from a similar incubation when 4-amino-5-imidazole carboxamide replaced the corresponding riboside.

Although the ability of the rabbit erythrocyte to carry out the reactions leading from a partially formed purine precursor to ATP has been demonstrated, its contribution to ATP formation in the circulating erythrocyte will remain obscure until plasma levels of the 5-amino-4-imidazole carboxamide riboside and related compounds are known.

While the ATP adenine specific activity curve is compatible with a turnover of at least part of the ATP molecule during the *in vivo* life span of the erythrocyte, as may well be the case, the negative results in the experiments concerned with *de novo* purine synthesis *in vitro* from the readily available small-molecule precursors suggested that perhaps another explanation was possible.

The alternative interpretation would not be the result of an actual turnover, but instead would result from a gradual decline in the ATP concentration in the erythrocyte during the aging process. Such a decline in ATP concentration could occur if, during the aging of an individual red blood cell, adenine nucleotides were lost and were not replaceable due to the lack of an intact purine biosynthetic mechanism. This loss would result in a decrease in specific activity in the ATP of the general cell population by dilution, since the newly formed erythrocytes would contain a higher ATP concentration than the partially depleted older cells. This could account for the exponential decrease in ATP adenine specific activity observed in the *in vivo* experiment. It may well be that the concentration of ATP in an individual erythrocyte is a function of the age of that cell, and that the loss of a compound of the importance of ATP might contribute to the demise of the cell *in vivo* as well as *in vitro*. There is as yet no conclusive evidence for this suggestion. However, it is known, that during storage *in vitro* the concentration of ATP in the erythrocyte diminishes, and that the resultant impairment of the glucose transport mechanism may be associated with the loss of viability of stored cells. Furthermore, Rapoport *et al.*[21] and Hofmann and Rapoport[22] have shown that the ATP content of the reticulocyte is about 2½ times that of the concentration of ATP in the average erythrocyte in the circulation. Although the reticulocyte loses its reticulum during the maturation process, there is no reason to presume the loss of the bulk of its ATP at that time. It is possible that the newly formed erythrocyte begins its travels in the circulation with 2½ times the ATP content of its average neighbor, and that during the life span of the erythrocyte the concentration of this compound might decrease to such a low level that essential aspects of the cell's metabolism would be halted. Such changes, in turn, would result in irreversible damage to the cell and, finally, to loss of viability and eventual destruction. The ATP of the erythrocyte would thus bear a relationship to the aging process *in vivo* and might be a determinant of the life span of the erythrocyte.

Studies currently in progress should provide information pertinent to the alternative interpretations that have been discussed.

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PROBLEMS CONCERNING THE KINETICS OF THE REACTIONS OF OXYGEN, CARBON MONOXIDE, AND CARBON DIOXIDE IN THE INTACT RED CELL

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In a recently published series of papers a quantitative appraisal has been made of the various factors concerned in the speed of uptake of oxygen and of carbon monoxide by the blood in the human lung (Forster *et al*,[1] Roughton *et al.*,[2] Forster *et al.*,[3] Roughton and Forster[4]). A brief summary of these investigations is presented in the first section of this paper as a prologue and aid to the main problem to be discussed herein: namely, the kinetics of the reactions of carbon dioxide in whole blood.

The Kinetics of O_2 and CO Uptake in Whole Blood

The following is the actual chain of processes involved in O_2 or CO uptake by the blood in the lung.

(1) Diffusion through the layer of pulmonary epithelium and the walls of the pulmonary capillaries, which separate the air in the minute lung alveoli from the blood plasma circulating through the capillaries.

(2) Passage through the layer of plasma surrounding the red cells of the plasma. This process is probably a composite one, consisting partly of diffusion and partly of mechanical mixing of the plasma.

(3) Diffusion through the red cell membrane.

(4) Diffusion through the finite thickness of the interior of the red cell plus simultaneous chemical combination with the hemoglobin contained therein.

It is convenient to regard each of these four processes as being in the nature of a "resistance" to the passage of the dissolved gas, the total over-all resistance being equal to the sum of these individual resistances taken in series. FIGURE 1a summarizes the combined results of the theoretical calculations and experimental determinations of the various resistances in the case of oxygen uptake; for the full details thereof the reader is referred to the papers cited above and to the review articles of Forster[5] and Roughton.[6] It has so far not been possible to evaluate the resistances due to processes 1 and 2 separately, but only to obtain their sum—this is shown in the figure as a stippled rectangle and is seen to represent about one half of the total resistance. The hatched rectangle, representing the red cell membrane (that is, process 3) corresponds to about one quarter of the whole resistance, as is also the case with the unshaded rectangle representing process 4, that is, diffusion and simultaneous chemical reaction velocity within the red cell. Process 4, in the case of oxygen, is only about one tenth as fast as the pure rate of chemical combination of this gas with hemoglobin in homogeneous solution: in the case of carbon monoxide, which at 37° C. combines homogeneously only with hemoglobin about one third as fast as oxygen does, the discrepancy is correspondingly less marked.

The actual value of r, the ratio of the rate in a layer of thickness b_1, in which

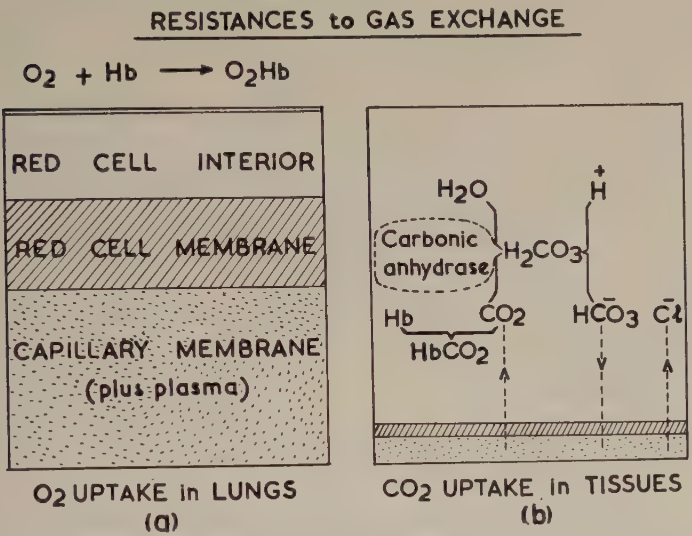

Figure 1. Relative importance of various processes in the over-all speed of O_2 uptake by the blood in the lungs. Heights of rectangles are proportional to the respective "resistances." For further explanation see text. (b) Similar diagram for CO_2 uptake in tissues. The stippled rectangle again represents the blood capillary membrane and the hatched rectangle the red cell membrane. Diffusion in this case is much less important than in "rate-determining" O_2 uptake.

the diffusion coefficient is D_1, to the rate in homogeneous solution is, during the initial phases of both processes, given by the equation (Roughton,[6])

$$r = \frac{\tanh \omega^*}{\omega} \tag{1.1}$$

where $\omega = b_1 \sqrt{\frac{k'[\mathrm{Hb}]}{D_1}}$ (1.2)

and k' = the velocity constant of combination of dissolved gas with the hemoglobin, a process that can be treated during the early phases as a bimolecular reaction (approximately). Insertion in EQUATIONS 1.1 and 1.2 of numerical values for b_1 (the average thickness of the red cell), for D_1 [the diffusion coefficient of O_2 or CO in hemoglobin solutions of the concentration found in the red cell (see Longmuir and Roughton[7])] and for k' (as measured by rapid reaction experiments in homogeneous hemoglobin solutions) enables r to be calculated for O_2 or for CO.

The above results are obtained from normal men at rest or in light activity and have so far met with fairly general acceptance among respiratory and clinical physiologists; their variation under pathological conditions is now being studied in many different laboratories, the three quantities that are generally the subject of determination being:

* Hyperbolic tangent.

(a) D_L, the over-all diffusing capacity of the whole lung, that is, the volume of O_2 or CO taken up in the lung in ml. per minute per mm. Hg gradient of the corresponding gas between the alveoli and the hemoglobin molecules in the red cells.

(b) D_M, the total diffusing capacity of the membrane separating the alveolar air and the blood plasma (defined in the same units as D_L).

(c) V_c, the total volume of blood (ml.) in the patent capillaries of the lung.

These 3 quantities (Roughton and Forster[4]) are related by the equation

$$\frac{1}{D_L} = \frac{1}{D_M} + \frac{1}{\theta V_c} \tag{2}$$

where θ = ml. of gas taken up by the red cells in 1 ml. of blood per minute per mm. Hg gradient of dissolved gas partial pressure between the exterior and the interior of the red cell.

The number of milliliters is determined by *in vitro* experiments on the rates of penetration of O_2 or CO into red cell suspension by the rapid reaction procedure of Forster *et al.*[1] From the value of θ it is possible to deduce the values of λ, which is defined as the ratio of the permeability of the red cell membrane to the permeability of the red cell interior (see papers by Forster *et al.*[1, 2]). Permeability is here defined as the ratio of the diffusion coefficient to the thickness of the phase tranversed so that

$$\lambda = \frac{D_2}{b_2} \Big/ \frac{D_1}{b_1} \tag{3}$$

where D_1, D_2 are the diffusion coefficients of the red cell interior and the red cell membrane, respectively, b_1, b_2 are the corresponding average thicknesses. For human red cells at physiological pH and 37° C. the value of λ is found to be about 1.5, alike for O_2, CO, and NO (Roughton[6]).

The Kinetics of CO_2 Transport in Whole Blood

Less is at present known of the relative kinetic importance of the various factors concerned in the rate of uptake of CO_2 by the blood in the tissues and of output of CO_2 from the blood in the lungs. This subject has been a profitable one in the past, for its study was directly responsible for the discovery both of the enzyme carbonic anhydrase and of the carbamino reaction of carbon dioxide with hemoglobin. At the present moment it is well worthy of further research, not only on account of the intrinsic interest of several more or less unexplored aspects of the problem, but also from the viewpoint of gaining a fuller understanding of the therapeutic effects of the drug acetazolamide (Diamox), which is a most potent inhibitor of the enzyme carbonic anhydrase, but *in vivo* seems to have a paradoxically slight effect on the kinetics of carbon dioxide transport within the circulating blood. The prime objective of the present paper is to make a new start on such a study by presenting preliminary data—some of them rather curious and quite possibly subject to later revision—on the rates of the individual processes schematically indicated in FIGURE 1b, which deals with uptake of CO_2 by the blood in passing through the tissue capillaries. All these processes are reversed in direction, of course, when the blood passes through the lung capillaries and yields up CO_2 to the air in the lung alveoli.

The first processes to be considered are the passages of CO_2 through the walls of the blood capillaries, through the plasma (wherein—owing to the absence normally of carbonic anhydrase—there is hardly any chemical combination of CO_2 during the one second or so that the blood takes, on the average, to pass through the patent tissue capillary), and through the red cell membrane. The actual diffusion coefficient of CO_2 through these various media, expressed in the standard physicochemical units of cm.^{2} sec.$^{-1}$, is probably about 0.85 of the corresponding diffusion coefficient of O_2 since, in liquid media, dissolved gases tend to diffuse at rates inversely proportional to the square root of their molecular weights:

$$(\sqrt{\text{mol. wt. of } O_2}/\sqrt{\text{mol. wt. of } CO_2} = \sqrt{32/44} = 0.8528)$$

The actual partial pressures of dissolved CO_2 in the blood and tissues are of the order of 50 mm. Hg, that is, about two thirds of the corresponding partial pressures of dissolved O_2. Since, however, the solubility coefficient of CO_2 in aqueous and lipoid media is about 30 times the solubility of O_2, the actual concentrations of dissolved CO_2 are of the order of $30 \times 2/3$, that is, 20 times the average concentration of dissolved O_2 in the blood. The gradients of dissolved CO_2 across the walls of the blood capillary, the plasma, and the red cell membranes tend correspondingly to be about 20 times greater than in the case of O_2. Therefore the times taken for diffusion across the barriers should be of the order of only one-twentieth the times for the analogous O_2 processes. The "resistances" of the capillary wall plus plasma and the red cell membrane, on this view, should be relatively only about one twentieth as great as in the case of O_2 uptake, and the heights of the stippled and hatched rectangles in FIGURE 1b are thus shown as only about one twentieth those of the analogous rectangles in FIGURE 1a. This way of stating the case, though not complete, is perhaps more satisfactory than that frequently adopted by physiologists when they say that CO_2 is 20 to 30 times more "diffusible" in aqueous fluids and body tissues than is O_2. This latter statement is true only if concentration gradients are measured in gas pressures, whereas actual amounts of gas diffusing are measured in volume units, for example, in ml.; this is often a convenient practice physiologically, but is apt to lead to confusion physicochemically, since physical chemists are accustomed to use the same units for measuring concentration gradients and amounts of diffusing material, so that their diffusion coefficients have the dimensions of cm.^{2} sec.$^{-1}$. Whichever convention is adopted, however, the net conclusion is the same: namely, that the "resistance" to diffusion of CO_2 from the tissues to the inside surface of the red cell membrane is only normally of the order of 3 to 5 per cent of the total "resistance" to CO_2 uptake. Such a fraction, though not inappreciable, is barely significant as a limiting factor.

After the CO_2 molecules have passed through the red cell membrane three further processes occur, the first two concurrently (FIGURE 1b): (1) reversible combination of CO_2 with hemoglobin in the carbamino form; (2) reversible combination of CO_2 with H_2O to form H_2CO_3 under the catalytic influence of carbonic anhydrase, which is present in mammalian red cells at a concentration of around 2.5 mg./cc. of red cells; and (3) exchange of HCO_3^- ions, formed within the red cell by ionic dissociation of H_2CO_3, with Cl^- ions from the plasma (the "chloride" or "Hamburger" shift).

We shall now discuss new, or recent data, on each of these three processes. Of these, process (1) is believed to be responsible for about 30 per cent of the physiological transport of CO_2, and processes (2) and (3) for most of the remainder. Still another type of binding of CO_2 has been postulated (the y-bound CO_2 of the blood) and has recently been investigated quantitatively by Milla *et al.*[8] Their data make it doubtful whether y-bound CO_2 is of much quantitative significance under normal physiological conditions in mammalian blood. Since, furthermore, nothing is at present known as to its kinetics, it will not be considered further in the present paper.

Within the red cell CO_2 must diffuse, of course, as well as react chemically according to processes 1 and 2, but calculations to be quoted later show that in both these cases the effect of diffusion, within a layer equal to the average thickness of the red cell, is only to reduce the homogeneous rates by less than about 12 per cent at most and probably by much less.

Carbamino combination of CO_2 with hemoglobin. It is perhaps appropriate to consider this reaction first, since it may be an earlier rapid reaction of CO_2 in the blood to appear developmentally than the carbonic anhydrase catalyzed reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$. Meldrum and Roughton,[9] it may be recalled, estimated the carbonic anhydrase content of the blood of a series of goat fetuses of various age, and found in the earliest cases an enzyme content equal only to about 0.5 per cent of the carbonic anhydrase of the blood of the adult goat.

The direct combination of CO_2 with hemoglobin was originally supposed, 30 years ago, and is still believed to belong to the general carbamino type of reaction, namely:

$$CO_2 + RNH_2 \underset{k}{\overset{k'}{\rightleftharpoons}} RNHCOOH \rightleftharpoons RHNC\overline{OO} + H^+ \qquad (4)$$

The pK of the substituted carbamic acid is thought not to exceed 6, so that at the normal pH of the interior of the mammalian red cell (pH 7.1 to 7.2) the carbamic acid will be completely ionized, or almost so.

Carbamino reactions of the type figured in EQUATION 4 are naturally fast reactions. TABLE 1 gives some values of k' (expressed in units of $M^{-1}\ s^{-1}$) for 3 amines studied by Faurholt[10]; somewhat more accurate, but confirmatory, figures for ammonia have recently been obtained by Pinsent *et al.*[11] There is seen to be a spread of one hundredfold in the value of k': calculations show that if the value of k' for hemoglobin were as low as that for ammonia, the speed of the reaction would be a rate-limiting factor under physiological conditions of CO_2 transport in the blood.

Roughton and Sirs have recently made some preliminary measurements of the speed of carbamino combination of CO_2 with sheep hemoglobin by an extension of a principle used by Keilin and Mann.[12] Washed sheep red cells

TABLE 1

Amine	k' (M^{-1} s^{-1})	
	at 20° C.	at 37° C.
Ammonia NH_3	300	1000
Glycinate ion $CH_2NH_2C\overline{OO}$	3,000	
Dimethylamine $(CH_3)_2NH$	30,000	

were treated with isotonic sodium nitrite for 5 minutes so as to convert the whole of the intracellular hemoglobin into methemoglobin. The sodium nitrite was removed by several washings with isotonic sodium chloride, and the red cells then hemolyzed by distilled water, the red cell ghosts being removed by centrifugation. The methemoglobin was diluted to about 0.25 g/100 ml. with CO_2-free distilled water and sulfanilamide (0.35 per cent) or Diamox was then added to inhibit the carbonic anhydrase content of the hemoglobin solution almost completely. The dilute methemoglobin solution was then mixed with a solution of CO_2 in a rapid reaction apparatus, and the rate of change of pH determined photocolorimetrically from the change of color of the methemoglobin, which acts as an indicator over the range 7.0 to 9.0 pH. Control experiments showed that this indicator change is, per se, complete within 0.002 sec. or less. From the initial rate of pH change the rate of the carbamino reaction can be calculated as in the following worked example of an experiment at 20° C:

Concentration of hemoglobin in mixed fluid = 0.125 gm./100 ml. = 7.5×10^{-5} Eq.

Concentration of sulfanilamide in mixed fluid = 0.01 M

Initial concentration of dissolved CO_2 in mixed fluid = 7.5×10^{-3} M

Initial pH = 7.6, pH after 0.05 sec. = 7.4

The number of H^+ ions required to produce a pH change from 7.6 to 7.4 can be calculated from the buffer power of the hemoglobin together with that of the sulfanilamide (pK = 10.4) over the pH range and the result comes out to be about 5×10^{-5} M.

We then have, during the first 0.05 seconds, approximately k' $[CO_2]$ $[HbNH_2] + k_u [CO_2] = 5 \times 10^{-5}$ M/0.05 sec. where $[HbNH_2]$ = concentration of free $-NH_2$ available for carbamino formation at the initial pH and temperature of the experiment = n [Hb] and k_u = velocity constant of the uncatalyzed reaction $CO_2 + H_2O \rightarrow H_2CO_3 = 2.2 \times 10^{-2}$ (M^{-1} s^{-1}) at 20° C.

$$\text{thence } k' \, n[Hb] = \frac{10^{-3}}{[CO_2]} - 2.2 \times 10^{-2} = 0.11$$

$$\text{thus } k' = \frac{0.11}{7.5 \times 10^{-5} n}$$

$$= \frac{1467}{n}$$

Over the pH range in question n is probably of the order of 0.5, giving k' for hemoglobin $\sim$ 3000, which is in line with the value for the glycinate ion at 20° C. as listed in TABLE 1.

Let us now examine, very approximately, the role of a reaction of this rate in the human respiratory cycle. In the red cell the hemoglobin is about 220 times as concentrated as in the worked example just quoted, so that if the rate of CO_2 combination is approximately proportional to the hemoglobin concentration (as is the case for O_2 and CO combination) the value of $k'n$ [Hb] in the red cell at 20° C. should be of the order of $220 \times 0.11 = 24.2$. At 37° C., if the effect of temperature is similar to that in the case of combination of CO_2 with NH_3 (see TABLE 1), $k'n$ [Hb] should be about 80.

During the respiratory cycle in normal resting men the carbamino-bound CO_2 within the red cell increases by about 1.4 mM when the blood passes from the arterial to the venous condition (see Roughton,[13] TABLE XI). The venous $[CO_2]$ = about 1.25 mM and the arterial $[CO_2]$ = about 1.07 mM. The minimum time t required for the formation of 1.4 mM carbamino-bound CO_2 should therefore be given very approximately by the equation

$$(1.25 - 1.07) \times 80 \times t = 1.4$$

whence $t \sim 0.1$ sec.

This time is of the order of one tenth that which the blood is usually supposed to take to traverse the tissue capillaries, so that, if the figures so far given are of the right order of magnitude, it appears that carbamino formation may be, at any rate to some extent, a rate-limiting step in CO_2 uptake by the blood in the body. Much further work is accordingly desirable on the kinetics of the CO_2-Hb combination and also on the converse process—the rate of dissociation of the carbamino hemoglobin compound —so as to enable analogous calculations to be made on the role of the carbamino reaction in the kinetics of CO_2 output in the lung. It is hoped that it may also be possible to carry out the rapid reactions pH measurements by means of Sirs' stopped flow-glass electrode method[14], thus permitting oxyhemoglobin and reduced hemoglobin to be investigated in place of the less physiological compound methemoglobin.

Assuming again at 37° C. a value for $k'n$ [Hb] in the red cell interior of 80, and values of $D_1 = 6 \times 10^{-6}$ cm.^{2}/sec., $b_1 = 7 \times 10^{-5}$ cm., we then have (see EQUATION 1.2)

$$\omega = 7 \times 10^{-5} \sqrt{\frac{80}{6 \times 10^{-6}}} = 0.256$$

$$\text{whence } r = \frac{\tanh\,(0.256)}{0.256} = 0.98$$

Diffusion within the finite thickness of the red cell should thus only reduce the over-all rate of carbamino combination by 2 per cent, in marked contrast with the effects of diffusion on the over-all rates of combination of O_2 and CO inside the red cell.

The activity of carbonic anhydrase inside the red cell. Various indirect estimates have been made during the past 25 years of the catalytic effect of the carbonic anhydrase present in the red cell. In these calculations it has been generally assumed that the effect remains proportional to the enzyme concentration over the whole range extending from dilute carbonic anhydrase solutions (0.1 to 1.0 mg./l.), in which the activity is usually measured, up to the concentrations found in mammalian red cells (2 to 3 gm./l.). On this basis Roughton has given figures ranging from 1500-fold[15] to 5000-fold[13] for the magnification, by the enzyme in the red cell, of the uncatalyzed rates of hydration of CO_2 and dehydration of H_2CO_3. The range is accounted for by the gradual growth in knowledge of the factors on which the activity of the enzyme depends. A more recent estimate, quoted by Hunter and Lowry,[16] gives a magnification factor of about 8000-fold for human red cells at 38° C. Clearly, however, the actual activity of the enzyme at the concentrations present in red cells should be measured directly rather than be extrapolated from data at enzyme concentrations only about one thousandth as great.

Five years ago W. W. Forrest,[17] in our laboratory, made some preliminary measurements of the activity of solutions of purified carbonic anhydrase at concentrations up to 1 gm./l., using for this purpose a modification of Chance's rapid reaction velocity apparatus.[18] FIGURE 2 shows the results of a typical experiment on the effect of various enzyme concentrations (plotted horizontally) on the rate of hydration of 0.02 M CO_2 when mixed with M/15 phosphate buffer, *p*H 7.8, at 20° C. The phosphate buffer contained paranitrophenol (*p*-NO_2-ϕ) in a concentration of about 10^{-4} M to act as a *p*H indicator

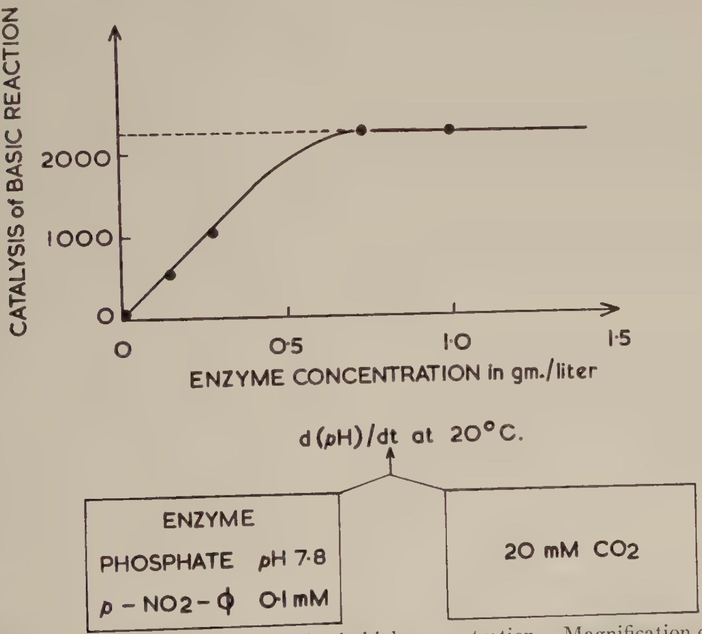

FIGURE 2. Carbonic anhydrase activity in high concentration. Magnification of the rate of hydration of CO_2 by phosphate buffer at purified enzyme concentrations ranging from 0.01 gm. to 1 gm./l., as measured by a rapid reaction *p*H method with paranitrophenol (*p*-NO_2-ϕ) as indicator.

of the progress of the hydration. This indicator, which changes from yellow to colorless in more acid *p*H—its *p*K being 7.1—, is a very suitable one for the purpose, for it has only a very slight protein error and has no detectable inhibitory (or activating) effect on the enzyme. Up to a carbonic anhydrase concentration of about 0.4 gm./l. the magnification of the rate of the basic reaction remains proportional to enzyme concentration, but the effect of the latter then tapers off, finally reaching a plateau at 0.7 gm./l. This effect was regularly reproducible and was also observed when the reverse process, that is, dehydration of carbonic acid, was similarly studied. If it also applies in the conditions met with in the red cell, it would be necessary to reduce current estimates of the activity of the enzyme therein, perhaps almost to the original figure of 1500-fold magnification given by Roughton[15] in 1935. Before doing so it is, however, equally necessary to make direct determinations of the enzymic activity of concentrated carbonic anhydrase solutions in the presence of hemoglobin at the concentration found in the red cells, for the hemoglobin may well have some additional effect on the enzyme activity. Indicator *p*H methods would scarcely be feasible for this purpose, but it might be possible to solve the problem by some adaptation of the thermal methods for following the velocity of rapid chemical reactions (Roughton[18]).

No certain theoretical basis has as yet been found for the effect shown in FIGURE 2. It may possibly be due to changes in aggregation of the enzyme in more concentrated solution, but further investigation is obviously desirable.

The values of ω (see EQUATION 1.2) corresponding to 1500-fold and 8000-fold magnifications of the uncatalyzed rate of hydration are 0.28 and 0.65, respectively. The corresponding values of r are 0.975 and 0.88, again pointing to a much slighter effect of diffusion within the red cell than in the case of the reactions of hemoglobin with O_2 and CO.

Speed of the chloride-bicarbonate interchange. This process is responsible for about 40 per cent of the physiological CO_2 transport in normal resting men; a comparison of its speed with that of the tempo of events in the blood capillary is therefore very pertinent. Dirken and Mook[19] were the first to investigate this problem, using for the purpose an ultrafiltration modification of the Hartridge-Roughton rapid reaction velocity apparatus. They found that the chloride-bicarbonate interchange, in suspensions of defibrinated ox red cells, was 90 per cent complete in about 1.3 sec. at 15° C. In recent years Dirken and Mook's apparatus has been modified by Paganelli and Solomon[20] and by Tosteson[21] for the study of the rate of exchange of isotope-labeled water and isotope-labeled chloride, respectively, between the inside and outside of the red cell. One of us (Rupp) has made a further adaptation of Tosteson's apparatus for the specific purpose of measuring the rate of chloride-bicarbonate interchange at 37 to 38° C.

Oxygenated bullock blood and serum saturated with CO_2 were driven by separate leads into a mixing chamber and thence down a rectangular channel observation tube, along the length of which were located 6 small millipore filters that provided ultrafiltrates from the flowing mixture. The chloride content of the ultrafiltrates was determined gasometrically by the method of Sendroy[22] to an accuracy of about ± 0.2 mEq. Cl. At the usual rates of flow

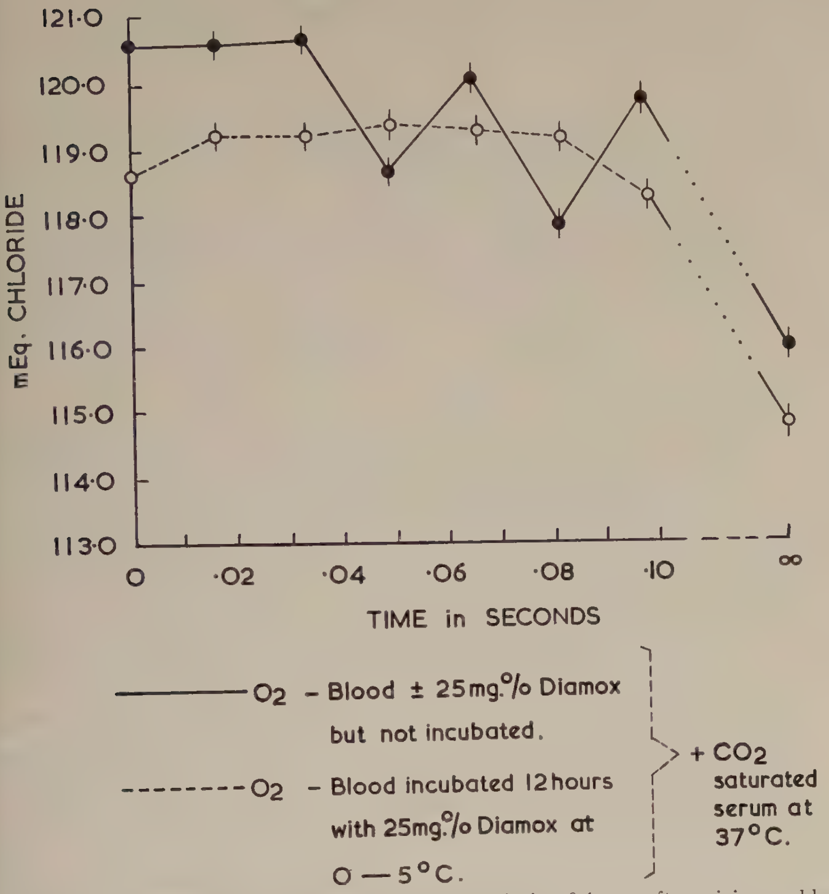

FIGURE 3. Changes in chloride content of serum during 0.1 sec. after mixing ox blood with CO_2-saturated serum at 37° C.

the times corresponding to the 6 ultrafiltrates were 0.016, 0.032, 0.048, 0.064, 0.080, and 0.096 seconds. With normal blood at 37° C. or with blood treated for about 1 hour beforehand with either of the carbonic anhydrase inhibitors, Diamox (25 mg./100 ml.) or sulfanilamide (125 mg./100 ml.), a remarkable periodicity in the chloride concentration is seen (see unbroken curve of FIGURE 3). If, on the other hand, the blood is incubated overnight at 0 to 5° C. with the same amounts of Diamox (or of sulfanilamide) and the experiment is repeated next day, all trace of the periodic phenomenon disappears. Unfortunately, the apparatus lacked sufficient filtration ports to allow for the determination of the course of the entire ionic interchange, which must obviously be the goal of further work in this field. The results, although thus far they are only preliminary, seemed to be quite repeatable: at present we have no satisfactory explanation of them and, until we have, it would clearly be premature to attempt to apply them to the conditions existing in blood capillaries. Equally clear, however, is the challenge that these preliminary results throw out for further work in the future.

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THE ELECTRON TRANSPORT SEQUENCE OF METHEMOGLOBIN REDUCTASE*

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The susceptibility of hemoglobin (Hb) to oxidation by molecular oxygen to form methemoglobin (MHb) during its normal function of undergoing reversible oxygenation with molecular oxygen to form oxyhemoglobin (HbO_2) may be represented by EQUATION 1:

$$\begin{array}{l} \mathrm{MHb} \\ \big\uparrow \mathrm{O_2} \\ \mathrm{Hb} + \mathrm{O_2} \rightleftharpoons \mathrm{HbO_2} \end{array} \qquad (1)$$

Quantitatively, the oxidation reaction is hardly significant, since essentially all of the hemoglobin molecules carry out the required oxygenation reaction without undergoing oxidation. However, even the small amount of oxidation, estimated at about 1 to 2 per cent of the total red cell hemoglobin per day (Lemberg and Legge), would represent a serious physiological problem if unchecked, since the cell eventually would contain largely methemoglobin, which is incapable of transporting oxygen to the peripheral tissues. While it is entirely possible that certain reducing agents in plasma (for example, ascorbic acid) could bring about the reduction of at least a portion of the continuously generated methemoglobin, it seems evident that some enzymatic mechanism also must exist in order to maintain the steady-state level of methemoglobin in the cell at less than 1 per cent. Such systems, called methemoglobin reductases, were first discovered and extensively studied by Kiese (1944). Evidence was presented that the erythrocyte contains two separate reductases, one dependent upon reduced triphosphopyridine nucleotide (TPNH) as its source of reducing power, and the other dependent upon reduced diphosphopyridine nucleotide (DPNH), as noted by Wendell and by Kiese (1944). TPNH may be generated by the operation of the glucose-6-phosphate dehydrogenase, while DPNH may be generated by lactic dehydrogenase in the cell. The present discussion will be concerned solely with the TPNH methemoglobin reductase.

A number of investigators have observed that the oxidation of glucose by intact erythrocytes is markedly stimulated (tenfold to twentyfold) by the addition of methylene blue (MeB), as noted by Harrop and Barron; by Michaelis and Salomon; by Warburg and Christian; by Wendell; and by Huennekens *et al.* (1957a). Actually, glucose is first converted to glucose-6-phosphate (G-6-P) by way of the hexokinase reaction, and it is the oxidation of G-6-P, mediated by the TPN-linked G-6-P dehydrogenase (EQUATION 2), that is responsible for the oxygen uptake (EQUATION 3):

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$$\text{G-6-P} + \text{TPN}^+ \rightleftharpoons \text{6-phosphogluconate} + \text{TPNH} + \text{H}^- \qquad (2)$$

$$\text{TPNH} + \text{H}^+ + \text{O}_2 \xrightarrow[\text{stimulated}]{\text{methylene blue-}} \underset{(\text{or } \text{H}_2\text{O})}{\text{TPN}^+ + \text{H}_2\text{O}_2} \qquad (3)$$

Kiese further showed that either oxygen or MHb could serve as terminal electron acceptors for the enzyme system catalyzing reaction (EQUATION 3).

Our own interest in the problem was initiated by a desire to understand this phenomenon, for it is unusual to find that a cellular electron transport system, presumably operating under physiological conditions, should be so markedly stimulated by the addition of an artifical electron carrier. Initially, we found that substrate amounts of TPNH were oxidized by molecular oxygen in hemolyzates of red cells, provided that MeB was added. The presence of Hb in the hemolyzate made it impossible to follow the disappearance of TPNH by conventional spectrophotometric methods, and we had to rely, instead, upon the more cumbersome manometric methods. Fortunately, this difficulty was overcome by the finding that hemolyzates could be treated with a mixture of ethanol and chloroform, which resulted in complete denaturation of Hb, but left the desired enzyme fully active in the aqueous fraction (Huennekens and Gabrio and Huennekens, *et al.*, 1957a). At this stage it was possible to change to a spectrophotometric assay involving the disappearance of TPNH as measured by the decrease in optical density. When TPNH, buffer, and enzyme are mixed together in a cuvette, the optical density at 340 mμ does not decline until a catalytic amount (0.03 μmole) of MeB is added; dissolved oxygen in the cuvette serves as the terminal electron acceptor. Using the customary techniques of protein fractionation (precipitation at *p*H 5.4, treatment with calcium phosphate gel, and fractional precipitation with ammonium sulfate) it was possible to bring the enzyme to a high state of purity. Based upon the original hemolyzate, the enzyme was purified about one hundred and eightyfold and was recovered in about a 30 per cent over-all yield (Huennekens *et al.*, (1957b).

The reductase is a red, heme-containing protein the absorption spectrum of which is shown in FIGURE 1; the heme group can assume three different oxidation states. From its absorption spectra the protein resembles Hb. However, the minimum molecular weight of the enzyme is 185,000 (based upon total heme and total iron determinations), whereas that of hemoglobin is 68,000. Furthermore, crystalline human hemoglobin possesses no activity as a methemoglobin reductase. Still other evidence for the nonidentity of these two similar heme proteins is the fact that they migrate differently upon chromatography on ion-exchange resins of the type described by Morrison and Cook or on starch block or paper electrophoresis strips (Lonn and Motulsky).

The purified enzyme was specific for TPNH; only about 20 per cent as much activity was observed with DPNH. No activity was observed when MeB or other auto-oxidizable dyes were replaced by any of the known flavin nucleo-

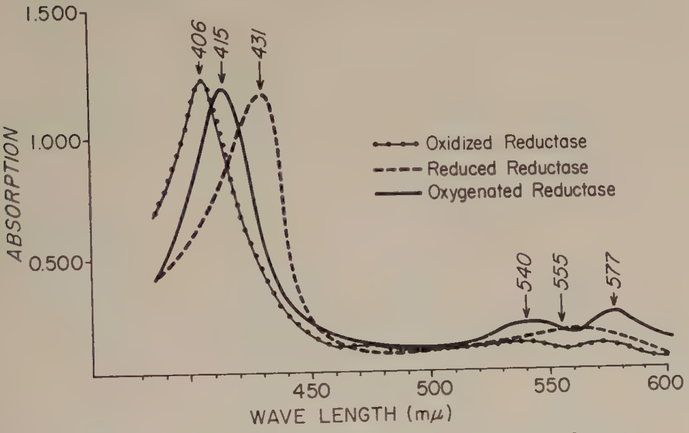

FIGURE 1. Absorption spectra of methemoglobin reductase.

tides, nor was any bound flavin ever observed in the enzyme at any stage in its purification. This is contrary to the suggestion of other investigators that the enzyme is a flavoprotein (Kiese, 1944; Gibson). As terminal electron acceptors, either oxygen, MHb, or cytochrome *c* could be used. Surprisingly, the greatest activity is observed with cytochrome *c*, although this substance is not a constituent of the mature erythrocyte.

As mentioned previously, the enzyme is a heme protein having characteristic absorption spectra. Three distinct oxidoreduction forms of the heme can be recognized by means of suitable chemical manipulations. Thus, treatment of the enzyme with ferricyanide leads to the oxidized form of the heme, while reduction of the enzyme with hydrosulfite in an anaerobic system leads to the reduced form. When the latter form is exposed to oxygen the reduced-oxygenated form of the heme is produced. The absorption spectra of these three forms of the enzyme are shown in FIGURE 1. It is of interest to note that heme proteins having absorption spectra similar to that of Hb have also been found in other tissues (Burris and Haas, Keilin and Wang, and Virtanen), and it is believed that these proteins may also function as respiratory carriers or terminal oxidases.

By means of the absorption spectra just presented, it has been possible to elucidate the sequence of electron transport during the oxidation of TPNH. In the experiment illustrated in FIGURE 2, substrate amounts of enzyme are used, and the experiment is begun by recording the absorption spectrum of the heme in the oxidized state. Upon addition of TPNH the absorption curve remains unchanged except for an additional band at 340 mμ due to the added pyridine nucleotide (curve A). When a catalytic quantity of MeB is added to complete the sequence, the absorption spectrum of the heme changes immediately to that of the reduced form (curve B). After all of the heme has been reduced, the MeB is finally reduced as shown by the decrease in the absorption maximum at 660 mμ (curve B′). If air is now admitted to the system, the absorption spectrum shifts to that of the reduced-oxygenated complex (curve

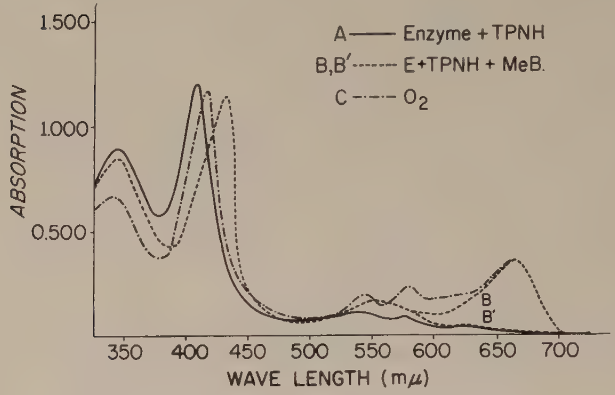

FIGURE 2. Sequence of electron transport.

C). This experiment demonstrates that the electron transport sequence in the enzyme under these particular experimental conditions is that shown in EQUATION 4:

$$\text{TPNH} \xrightarrow{e^-} \text{MeB} \xrightarrow{e^-} \text{Heme} \xrightarrow{e^-} O_2 \tag{4}$$

Quite apart from the fact that the naturally occurring cofactor, which is replaced in the assay system by MeB, is still unidentified, there are several other aspects of the electron transport sequence which need clarification. For example, it is not yet known whether the terminal electron acceptors, which are external to the enzyme, are reduced directly by the reduced heme on the enzyme or whether the reduced methylene blue (or its naturally occurring counterpart) can interact directly with the external terminal electron acceptor. Second, when oxygen is the terminal electron acceptor, it is not known whether the product is H_2O or H_2O_2. After the reducing power from the TPNH is transferred through the enzyme to reduce the enzyme-bound heme, this latter moiety may then interact with oxygen to produce the oxygenated complex of the reduced heme. Does this complex (possibly four hemes combined with a single oxygen molecule) then undergo a dismutation reaction whereby the hemes are oxidized to the trivalent state, and the oxygen is reduced to water? In terms of its interaction with oxygen as a terminal acceptor, methemoglobin reductase is somewhat similar to cytochrome oxidase.

Even under optimal conditions the turnover number of the enzyme is small; from various lines of evidence we have estimated that it is about 100. Turnover numbers of this low order of magnitude are, of course, not uncommon in enzymatic reactions, but are somewhat unusual with electron-transport enzymes. However, calculations have shown that the amount of this enzyme present in the red cell, even functioning with its low turnover number, can adequately dispose of the amounts of MHb that are formed each day. The low turnover number of the enzymatic reaction does, however, pose another problem inasmuch as the blank reactions, due to *chemical* interaction of the various substances, must be taken into account. For example, MeB and

TPNH interact chemically, and the rate of this reaction is greatly accelerated (from fivefold to tenfold) in the presence of light, which is absorbed by the dye. In fact, a good chemical model for a TPNH oxidase can be constructed in this manner. When TPNH and MeB are mixed together in a cuvette TPNH begins to disappear at a linear rate. The MeB remains in its oxidized (blue) state, indicating that the air-oxidation of reduced (colorless) MeB exceeds the rate of reduction of the dye by TPNH. If, however, the cuvette is irradiated by light of 660 mμ in the spectrophotometer, or even held in sunlight, the rate of TPNH disappearance is greatly increased, and the dye now becomes completely bleached. Since reduced MeB is also capable of interacting chemically with MHb or with cytochrome *c*, it is thus possible to prepare a nonenzymatic TPNH methemoglobin reductase. Fortunately, the rate of these chemical reactions is of an order of magnitude slower than their enzyme-catalyzed counterparts; however, corrections must always be made for such chemical blanks.

At times, after the ethanol-chloroform step and during the preparation of reductase, we observed a limited ability of the enzyme to function in the absence of MeB. Although this activity was small in comparison to that with addition of MeB, it was significant and reproducible. Upon further purification, however, the enzyme lost all ability to operate in the absence of added MeB. These observations suggested that the enzyme originally contained some naturally occurring cofactor that participated in the electron-transport sequence, but that this factor was readily detached during the manipulations of purification, thus necessitating the inclusion of an artificial carrier, MeB.

In order to undertake the isolation of this unknown "cofactor," we turned to beef blood as a starting material, since larger quantities of enzyme were obviously needed. The same purification procedure was used, and it was found that the highly purified beef reductase was very similar in its properties to the human enzyme. However, in the crude form (that is, after the ethanol-chloroform step) the beef enzyme contained much more of the "natural cofactor" than did the human enzyme and, therefore, was less stimulated by MeB. In fact, certain preparations of the crude beef enzyme were not stimulated at all by the addition of MeB. However, if the beef enzyme was treated with ammonium sulfate or passed through Dowex-1 columns (chloride form), an absolute requirement for MeB was elicited.

The absorption spectrum of the crude beef enzyme showed considerable quantities of bound material in the region of 260–300 mμ. These bound materials were quite resistant to exhaustive dialysis, but were removed by ammonium sulfate fractionation or by passage of the preparation through Dowex-1 columns. It would be premature to equate the "natural cofactor" with the bound ultraviolet-absorbing material, but there is no doubt that crude enzyme preparations, which do not require MeB, will serve as good sources for isolating the "natural cofactor."

A further complication in our understanding of the electron transport sequence arose when we investigated more closely the ability of the enzyme to use MHb, rather than oxygen, as the terminal acceptor. It had been noticed repeatedly that some preparations of the crude enzyme (containing "natural

TABLE 1

EFFECT OF Fe^{+++} ON METHEMOGLOBIN REDUCTION

Enzyme	Fe^{+++}	Versene	ΔE_{576}*
−	+	−	0.000
+	−	−	0.055
+	−	+	0.000
+	+	−	0.265
+	+	+	0.000

* Change in optical density at 576 mμ over a 10-min. period.

cofactor," and not requiring MeB) were still unable to reduce MHb, but retained an unimpaired ability to reduce oxygen. We searched for some factor that might restore to this enzyme its ability to use MHb as a terminal acceptor. For an assay system we turned to the reduction of MHb, which can be followed spectrophotometrically either by the disappearance of its absorption maximum at 630 mμ or by the appearance of the oxyhemoglobin absorption at 576 mμ.

It was found that ionic iron could provide the desired electronic linkage. As shown in TABLE 1, the addition of Fe^{+++} to an enzyme in the first stage of purification (that is, following ethanol-chloroform treatment of the hemolyzate) and in the absence of MeB, produces about a fourfold stimulation in its ability to reduce MHb. This effect is abolished completely when versene, a strong chelating agent, is added; in fact, versene also inhibits the original activity shown by the enzyme in the absence of added iron. Several other metals (Mg^{++}, Ca^{++}, MoO_3, and Mn^{++}) were all inactive. Since Fe^{+++} forms insoluble precipitates of hydrated ferric hydroxide at *p*H 7.5, it is necessary to add some complexing agent in order to keep the iron in solution. Citrate, adenosine triphosphate (ATP), and adenosine diphosphate (ADP) can be used for this purpose. Adenosine monophosphate (AMP) and inorganic phosphate are ineffective, while versene and pyrophosphate apparently bind the iron so tightly that it is unable to function in the enzymatic system. These experiments recall Neuberg's earlier observations that ATP and similar compounds may function, on occasion, as chelating agents in order to keep insoluble metals in solution (Neuberg and Mandl). When either ferric citrate or ferric ATP is used in the enzymatic system, the optimal concentration is 5×10^{-4} M. We have been able to show, moreover, that the addition of Fe^{++} to MHb in the presence of the enzyme produces a very rapid reduction of the external acceptor, while in the absence of the enzyme these substances interact at a slower rate.

Having summarized the general properties of our enzyme preparation, it may be pertinent at this point to contrast them with those described by Kiese (1957), who has used similar methods of fractionation. Kiese's enzyme contains some bound flavin nucleotide identified as FAD, and has no bound-heme moiety; also, it will not react with MHb as an acceptor. The reasons for these discrepancies between the two preparations are not presently understood, although the complexity of the electron-transport system between reduced pyridine nucleotide and final acceptor, and the possibility that the enzyme may be subdivided into smaller units, each representing a portion of the original transport sequence, may offer at least a partial explanation.

It may be of interest to discuss briefly the hematological disorder, methemoglobinemia, in terms of the above findings. It has been recognized recently that this disease, which is characterized by steady-state levels of MHb as high as 30 to 50 per cent, has two distinct etiologies. In the first of these the Hb in the cells is abnormal in that it is more susceptible than normal Hb to oxidation (Kiese *et al.* 1956). We have examined the blood of one such patient and have found that the addition of ferricyanide to hemolyzates of this blood causes MHb to appear at a rate 3 to 4 times as fast as that observed with normal bloods. Furthermore, the absorption spectrum of the resultant MHb is also abnormal, having its absorption maximum centered at 625 mμ instead of 630 mμ. The second type of methemoglobinemia may be considered more as an enzymatic defect. It is found in patients whose Hb is normal and is oxidized only at the usual slow rate by molecular oxygen in the cell, but the enzymatic apparatus for reduction of MHb is somehow impaired. The fact that such patients have been routinely treated by the infusion of massive amounts of methylene blue (Eder *et al.*) suggests that at least a portion of the enzymatic system is functioning. The continued search for the enzymatic defect in this type of methemoglobinemia continues to be a challenging problem.

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THE PERMEABILITY OF RED CELLS TO WATER AND IONS

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The cellular membrane of the human red cell is composed largely of proteins and lipids; in addition, calcium is believed to be present.[1] The lipids, mostly phospholipids, comprise about 35 per cent of the membrane by weight, and also include cholesterol and neutral fat.[2,3] Substances may enter the cell either by active or passive processes, which may be distinguished on the basis of their requirement for energy. Following Rosenberg,[4] transport may be classified as active or passive, depending upon whether it is up or down an electrochemical potential gradient, as shown in FIGURE 1. This definition has been extended by Ussing,[5] who also includes as active any transport down an electrochemical gradient which proceeds at a rate faster than that of simple diffusion and requires a supply of energy. Both active and passive processes play an important role in red cell physiology.

There are two routes of passive entrance into red cells as shown schematically in FIGURE 2. The membrane, as will be discussed below, is believed to be pierced by aqueous channels, through which small, neutral or negatively charged particles may penetrate. Another route is open to lipid soluble molecules, either large or small, which may enter the red cell by dissolution in the membrane. Oxygen and carbon dioxide are important examples of small molecules in this class. The rate of entrance of larger lipid-soluble molecules appears to be dependent, not only on their lipid solubility, but also on their molecular size;[6] this presumably reflects differences in their diffusion coefficients within the cellular membrane.

It is instructive to compare the rates of cation and anion entrance into the cells. Potassium has been shown by a number of investigators[7-9] to enter the red cell very slowly, requiring about 45 hours for the exchange of one half of the potassium in the red cell. Chloride, on the other hand, enters the red cell very rapidly. The half time for exchange of intracellular chloride has been estimated as 0.11 sec. by Luckner and Lo-Sing[10] and 0.24 sec. by Tosteson.[11] The relationship between potassium and chloride fluxes is shown diagramatically in FIGURE 3. Since they have almost the same mobilities in free solution, 7.6×10^{-4} cm.^{2}/volt sec. for potassium and 7.9×10^{-4} cm.^{2}/volt sec. for chloride, some factor other than free diffusion must be invoked to account for the millionfold difference in the half time of exchange. It is reasonable to assume that the channels in the membrane contain positive charges, thus impeding the passage of positively charged substances and accelerating the passage of negatively charged ones. Using the equations given by Meyer and Sievers[12] for pores of large diameter, it is possible to estimate that a charge density in the channels of 175 mols./l. would be required to provide the millionfold difference in half time. Such a charge density is equivalent to about 3 charges for every water molecule present, and consequently cannot be achieved. When the pores are small, however, the charge density effect is more complex and no equation has been given by Meyer and Sievers to take quantitative account of the additional hindrance provided when passage through the membrane is restricted to a small pore. Furthermore, as Sollner has pointed out,[13] a single charge can block a small pore effectively; it would therefore seem likely that small positively charged pores play an important part in restricting the diffusion of potassium.

Since the positive charges on the inside of the channels discriminate effectively against potassium, a mechanism other than diffusion is required to account for potassium entrance into the cells. Potassium is present in the

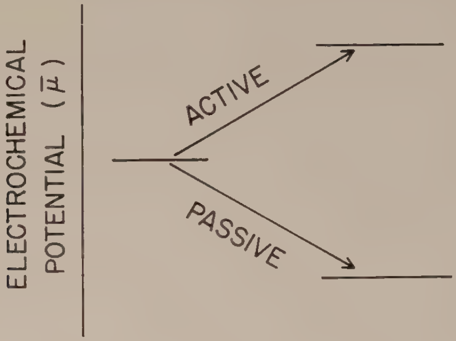

FIGURE 1. Active and passive transport.

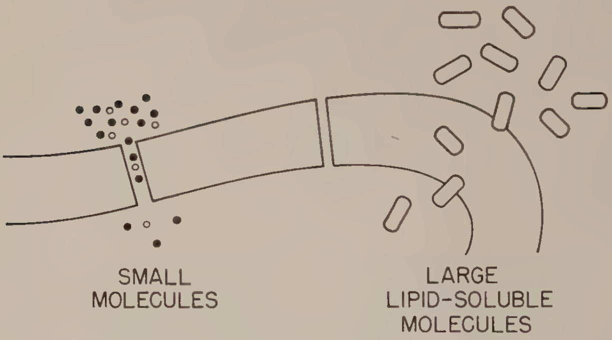

FIGURE 2. Passive entrance into red cells.

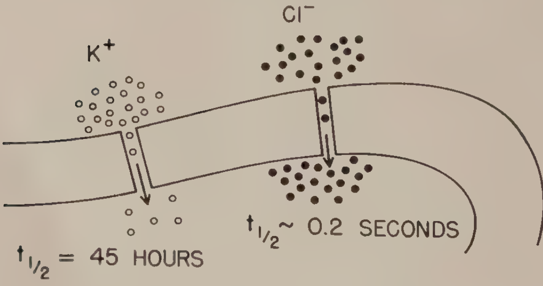

FIGURE 3. Ion entrance into red cells.

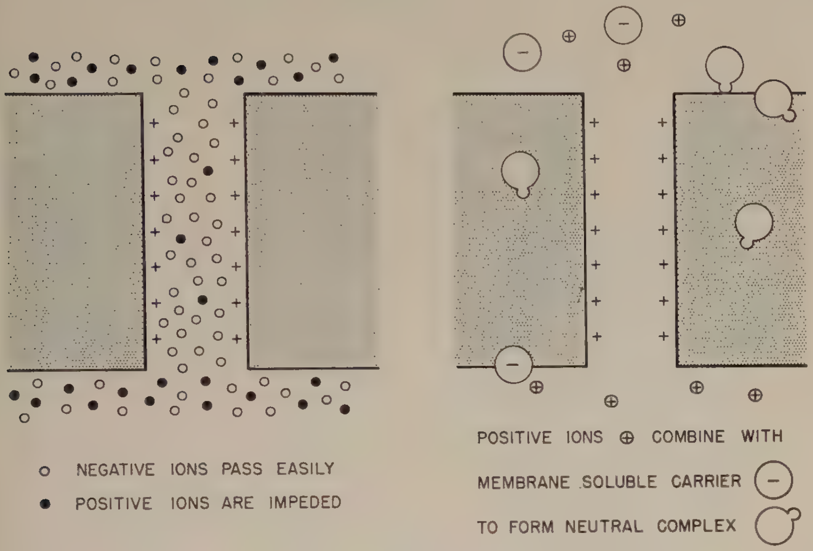
○ NEGATIVE IONS PASS EASILY
● POSITIVE IONS ARE IMPEDED
POSITIVE IONS ⊕ COMBINE WITH
MEMBRANE SOLUBLE CARRIER ⊖
TO FORM NEUTRAL COMPLEX

FIGURE 4. Positively charged pores in cellular membrane.

cells at a concentration of about 140 mmoles/l., about 27 times greater than its concentration in the plasma, about 5 mmoles/l. Thus, any net transport must be active unless the intracellular potential is at least 80 mv. negative with respect to the plasma potential. Such a potential difference, as will be discussed below, is greater by an order of magnitude than that actually observed. Consequently, a carrier mechanism has been proposed to account for the entrance of potassium into the cell.[7, 14] The cation is considered to enter into a reaction on the cell surface with a negatively charged lipid-soluble carrier which transports it into the cell, where the complex dissociates with the release of potassium, as shown schematically in FIGURE 4. Phospholipids, which are present in the cellular membrane, have been suggested as possible carriers.[15, 16]

The chloride distribution is considered to be passive. Its concentration inside the cell is 0.69 times the extracellular concentration,[17] which is the same figure found for the ratio of bicarbonate concentration after correction for the carbamino groups. Thus, one would be constrained to assume either that identical mechanisms operate for chloride and bicarbonate transport or that both are distributed as passive ions according to the electrochemical potential inside the cell. On this latter basis, it has been calculated[7] that the interior of the cell should be 9.9 mv. negative with respect to the plasma. Recently, Marsden and Neihoff[18] have made preliminary measurements of red cell potential. Their figures suggest that the intracellular potential is in the neighborhood of 5 to 10 mv. negative, in agreement with the passive distribution of chloride and bicarbonate. These considerations indicate that the intracellular environment is closely controlled with respect to cation content, but is dependent on the extracellular ionic environment with respect to distribution of small anions. Such specificity may be of interest in connection with the specific cation requirements of many enzymes.

Experiments carried out with the cardiac glycoside, ouabain, provide information about the number of available surface sites on which potassium may couple to the carrier. Ouabain has been shown to have no effect on red cell glucose metabolism[19, 20] and is presumed to act on the first step of potassium entrance, presumably on the membrane surface. Studies of the kinetics of this inhibition have led to an estimate of the number of effective sites on the surface. Maximum estimates, made independently by Glynn[20] and Solomon *et al.*,[21] lead to figures of 7.2 to 72 sites per square micron of red cell area. It appears, therefore, that the surface is sparsely populated with sites, having one site for every 10^6 to 10^7 Å^2 of cell surface. Consequently, the potassium flux is approximately 10^4 to 10^5 atoms per hour per site, equivalent to 3 to 30 atoms per second. The ouabain inhibition data may also be used to show the strength of the coupling between potassium and site. The association constant has been found to be 860 l. blood/mole which represents a much weaker attraction than that for ouabain, for which the association constant is 5.9×10^7 l. blood/mole.

It is possible to obtain indirect information about the radius of the pores in the red cell membrane by making measurements of water entrance under a diffusion gradient and under an osmotic pressure gradient.[22, 23] As shown in FIGURE 5, the former of these measurements leads to an approximate estimate of the pore area divided by the pore length and the second to an approximate estimate of this same factor times the square of the radius. It has been necessary to use the rapid flow technique to obtain measurements immediately after the initiation of each specific process, and it has been found that the half time for the diffusion exchange in human cells is about 4.1 msec., whereas the

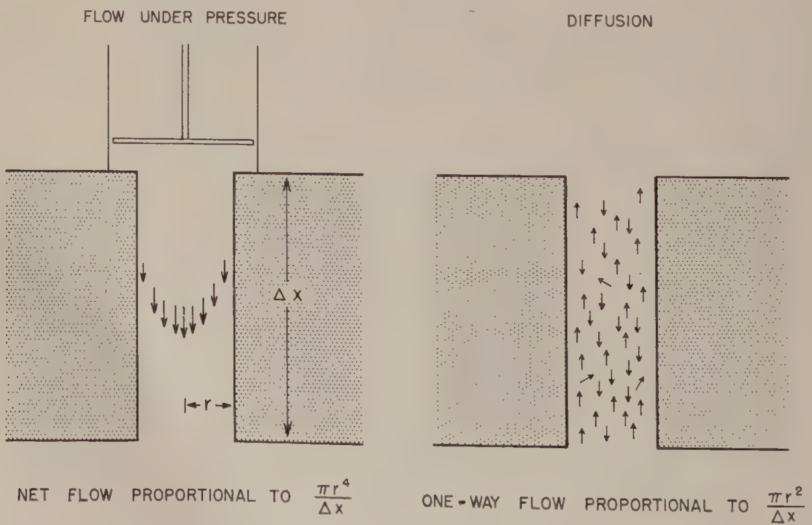
FLOW UNDER PRESSURE
DIFFUSION
Δ X
r
NET FLOW PROPORTIONAL TO $\frac{\pi r^4}{\Delta x}$
ONE-WAY FLOW PROPORTIONAL TO $\frac{\pi r^2}{\Delta x}$

FIGURE 5. Passage through pores.

TABLE 1

WATER FLOW ACROSS HUMAN RED CELL MEMBRANE[23]

	$\frac{\text{Ml. } H_2O}{\text{Sec., cm. } H_2O\text{, red cell}}$
Diffusion gradient flow	0.62×10^{-14}
Osmotic pressure gradient flow	1.5×10^{-14}

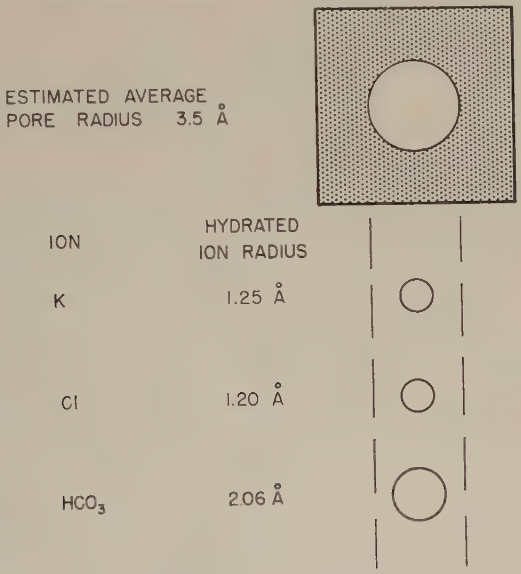

FIGURE 6. Red cell pore.

half time for water entrance under an osmotic pressure gradient is measured in tenths of a second. These figures may also be expressed in terms of water entrance under the same chemical potential gradient. For this purpose, it has been necessary to translate the diffusion gradient into an equivalent concentration gradient, making use of van't Hoff's law. As shown in TABLE 1, water entrance under an osmotic pressure gradient is more than double that under a diffusion gradient. The difference in these two figures has been interpreted by Koefoed-Johnsen and Ussing[24] as indicative of the presence of water-filled channels inside the membrane. The equivalent pore radius has been calculated by Paganelli and Solomon[23] as 3.5 Å, which may be compared, as shown in FIGURE 6, with the hydrated ionic radius* of K, which is estimated to be 1.25 Å, and Cl, estimated to be 1.20 Å. On the same basis the hydrated ionic radius of HCO_3 would be 2.06 Å; this ion should therefore cross the membrane more slowly than does Cl.

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* Calculated as described by Solomon.[25] The ionic radius is not to be considered as having a real meaning, being rather the imaginary radius that a hydrated ion would have if it were spherical, had a uniform charge distribution, and moved in an infinitely dilute solution; nevertheless, the ionic radius does serve as a qualitative index of hydrated ionic size.

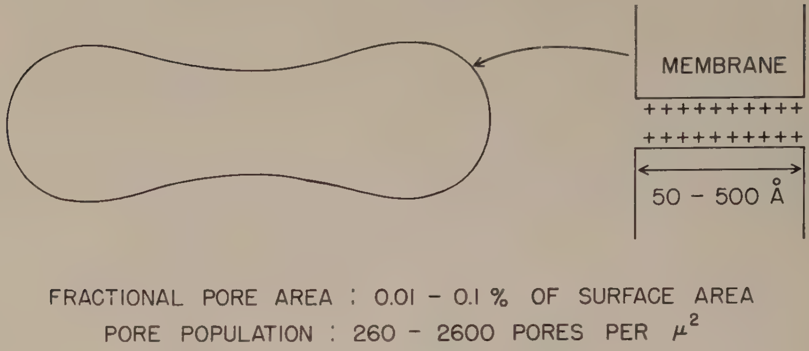
MEMBRANE
++++++++++
++++++++++
50 - 500 Å
FRACTIONAL PORE AREA : 0.01 - 0.1 % OF SURFACE AREA
PORE POPULATION : 260 - 2600 PORES PER μ^2

FIGURE 7. Pores in human red cells.

These measurements may be used also to make an estimate of the fraction of the red cell surface occupied by pores. For this purpose, it is necessary to make an assumption as to the path length through the membrane. Since estimates of membrane thickness vary considerably, it is desirable to calculate two fractional effective pore areas, on the basis of a minimum path length of 50 Å and a maximum of 500 Å, leading to pore areas of 0.01 per cent to 0.1 per cent of the cell surface as shown in FIGURE 7. The water permeability of the red cell, as pointed out by Jacobs,[26] is larger than that known for any other cell. It seems somewhat surprising that this permeability can be accounted for on the basis of a cellular membrane of which less than 0.1 per cent is available for water entrance.

FIGURE 8, which is based on some calculations made by J. L. Oncley (personal communication) from published data, shows schematically the relationship between the sizes of certain intracellular and extracellular proteins and the pore radius. It seems clear that enzymes, as such, are unable to enter red cells passively. It is also quite unlikely that such large molecules could enter

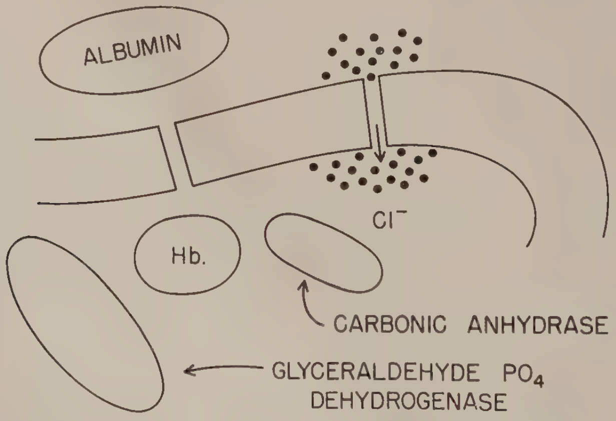

FIGURE 8. Entrance into red cells. The pore radii in this figure have been exaggerated for clarity.

the red cell by an active transport mechanism, unless by an infolding of the cellular membrane as, for example, in pinocytosis. Presumably the intracellular enzymes are synthesized within the cell either during the process of cell development or during its subsequent life.

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Part III. Enzymes in Blood Coagulation and in Platelets

IS THROMBIN THE ONLY ENZYME INVOLVED WITH PROTHROMBIN ACTIVATION?*

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The experimental work supporting the view that thrombin is the activator of prothrombin was described in papers from our laboratory ten years ago.[1, 2] Why, then, does this idea seem so surprising today? I think there are several reasons. First, most but not all of our demonstrations were concerned with prothrombin activation in 25 per cent sodium citrate solutions, and some of my readers may be inclined to feel that the "unphysiological conditions" make these experiments of consequence only in another domain. Second, much attention was attracted to the development of oral anticoagulants and, since mind is so constituted that attention is fixed on one phenomenon at a time, prothrombin activation was neglected. This was a peculiar neglect that, in most instances, consisted of a "brief review of the blood-clotting mechanism." This mechanism was admittedly not understood, but a plausible notion is helpful even if it obscures an accurate one. Third, clotting factors were discovered in many laboratories, including our own. Preoccupation with these developments became common. I once remarked that students of hemorrhagic diseases have "factorphilia" and will not easily find a cure. Some of these factors are previously unrecognized properties of prothrombin, others are not, and some factors are being deleted, the evidence for them being inadequate. For each one the claim has been made that it is essential for the activation of prothrombin.

How many essential events are there in the conversion of prothrombin to thrombin? I do not know; perhaps no more than one, but I report my view that no factor besides thrombin is needed to do this work. In other words, I deny that these several factors are essential, and I see them function in support of the work thrombin does. In this way I can be comfortable with factorphilia, for there may be all kinds of circumstances and combinations of factors that support or retard the activation of prothrombin. This introduces the unifying point of view that all procoagulants and anticoagulants, be they ever so numerous, support or inhibit the activation of prothrombin by thrombin. Thus, simplification is one of the consequences of this way of considering our presenting facts.

The first experiment to which I call attention was performed in 1948.[1] My associates and I had obtained excellent preparations of purified bovine prothrombin and also thrombin of high quality. About 13,000 units of pro-

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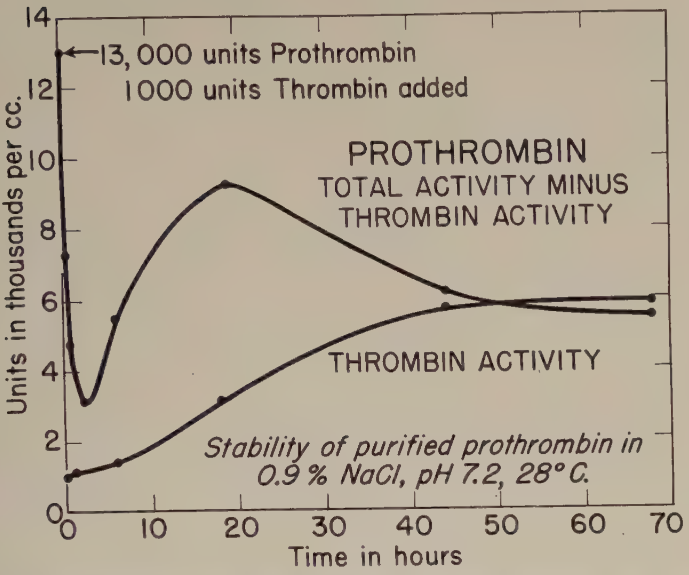

FIGURE 1. Prothrombin mixed with thrombin undergoes alterations and partially converts to thrombin.

thrombin were mixed with 1000 units of thrombin (FIGURE 1). During the first 4 hours the prothrombin becomes refractory to the analytic reagents used for measuring prothrombin concentration, then a peculiar change occurs. After 4 hours the prothrombin regains its capacity to become converted to thrombin and, at the same time, considerable free thrombin appears in solution. The experiment ended at 70 hours, when most of the original prothrombin or thrombin could be accounted for either as prothrombin or as thrombin. I conclude that prothrombin is activated by thrombin in the absence of any other known procoagulants. In this instance activation consists of at least 2 alterations that occur before thrombin is formed.

I do not know how to create a better perceptual image of this fundamental observation. However, I often hear the question: How do you know that some impurity in your preparations does not account for the results? My whole effort in regard to prothrombin has been and continues to be directed toward reducing these possibilities to a minimum so as to achieve an accurate perspective. I am convinced, however, that a sincere creator of a protein preparation can never avoid admitting the *possibility* of the existence of significant quantities of associated material. Thus, I might plead that there is only one molecule of "impurity" per mole of preparation and have my skeptic ask whether that is significant; I do not know whether each methyl group or hydrogen atom occupies the same position in all prothrombin molecules that come from the metabolic production line. We used prothrombin preparations of almost the same quality that is procurable today; however, the thrombin was not as free of other substances. Today we can obtain thrombin that is homogeneous by several methods of analysis. Our experiment was therefore performed with the best thrombin now available. The result is a strengthening and broadening of the previous point of view.

A year later I performed the second experiment of importance to our theme.[2] Prothrombin was dissolved in 25 per cent sodium citrate solution and it slowly transformed to thrombin (FIGURE 2). The process is autocatalytic, being initiated or accelerated by thrombin. The transformation can also be accelerated by certain sulfonamides or inhibited by others or by soybean trypsin inhibitor, as well as by substances in serum or plasma. When this discovery was made I was able to see clearly, for the first time, that prothrombin contains all that is required to have thrombin, that thrombin is activator under these conditions, and that all other substances concerned with prothrombin activation must therefore have the role of catalysts. There are decoys in the literature that distract from this view. I examine them more closely and let them go. For instance, there is the observation that this is happening under nonphysiological conditions and hence may be of little importance. My world in that I live in me is not divided into the physiological and nonphysiological. My mental material, which is my only material, is always physiological.

As thrombin is derived from prothrombin under certain conditions, its capacities to activate fibrinogen and to hydrolyze synthetic substrates are found in equal proportion. However, when it is left standing or treated with ninhydrin the clotting power may be lost entirely, while its esterase activity remains in appreciable concentration. In 0.05 M calcium chloride solutions

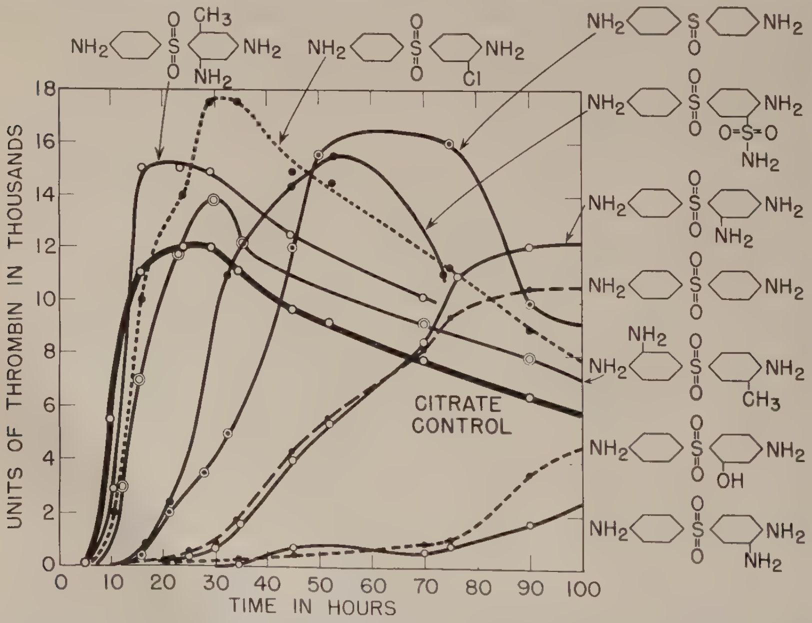

FIGURE 2. Prothrombin dissolved in 25 per cent sodium citrate solution changes to thrombin. After activation, activity again declines. Certain sulfonamides, in low concentration, inhibit the activation while others accelerate it.

prothrombin activates to yield first and, later predominantly, esterase activity.[3] In this manner material can be derived from prothrombin *to clot blood* or, when derived in other ways, *to dissolve fibrin clots*. I find it exciting to see a prothrombin derivative doing the opposite of what we were accustomed to expect from prothrombin. There is even a possibility that the esterase activity of blood is due to structures derived from prothrombin. Since the esterase activity can be developed preferentially, we assumed that the reverse likewise might be possible. We found conditions where the clotting activity is not altered, but where the esterase activity is depressed or perhaps preferentially occupied. The first condition noted was precisely one of those known to favor activation of prothrombin.

Concentrated sodium citrate solutions have the peculiar property of depressing the esterase activity of thrombin without changing its capacity to clot fibrinogen (FIGURE 3). Since prothrombin activates in sodium citrate solution, it is possible that the relative clotting and esterase strength of each new thrombin molecule is thrown out of balance, the clotting function being the one concerned with prothrombin activation. There might be an advantage in having the esterase quality out of the way, so to speak. In any event esterase thrombin itself does not serve as prothrombin activator, nor does it serve as Ac-globulin activator.

Platelet factor 3 is a lipoprotein found in platelets, and is one of the important procoagulants in the activation of prothrombin. We have developed

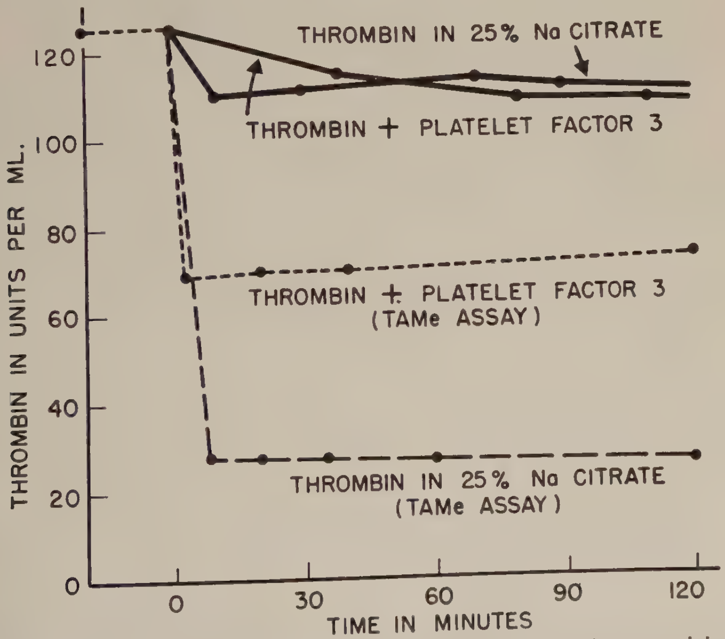

FIGURE 3. Purified biothrombin preparations are altered in solutions containing 25 per cent sodium citrate or purified platelet factor 3. The esterase activity is depressed without altering the clotting activity.

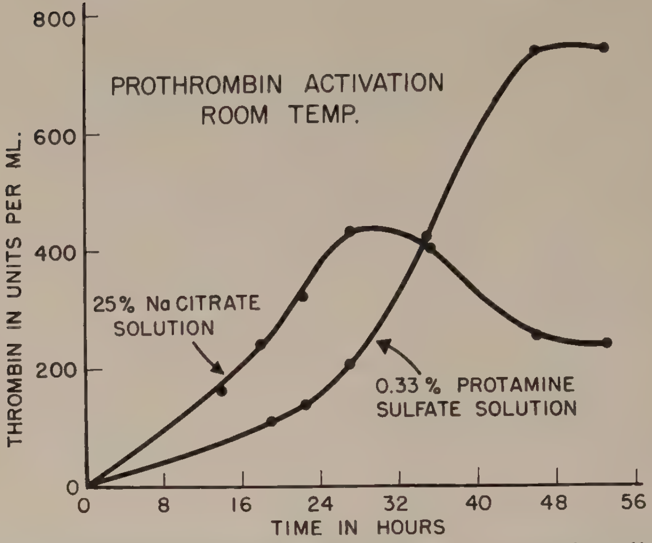

FIGURE 4. Purified prothrombin activates in 0.33 per cent protamine sulfate solution. All the substrate is converted to thrombin, whereas the yield is less in 25 per cent sodium citrate solution.

methods for obtaining excellent concentrates of it and have studied its function in the activation of prothrombin. This substance also depresses or occupies the esterase activity of thrombin. I "complained" above that some authors regard prothrombin activation in sodium citrate solution as "unphysiological," which is very interesting in itself, but perhaps of little importance to physiology. Notice, now, the feature that concentrated sodium citrate and purified platelet factor 3 have in common: both depress the esterase activity of thrombin.

There is another substance that depresses the esterase activity of thrombin; namely, protamine sulfate. Purified prothrombin was then placed in a solvent consisting of 0.9 per cent NaCl and 0.33 per cent (w/v) protamine sulfate at *p*H 7.2. Within two days all of the prothrombin became thrombin (FIGURE 4) The activation curve is one that is characteristic of autocatalysis. The yield is even better than in sodium citrate solution.

Consider the formation of an enzyme substrate complex.[4] Three experiments were performed (FIGURE 5). In the first experiment thrombin (9 units/ml.) was dissolved in physiological saline solution together with 0.01 M *p*-toluenesulfonyl-L-argine methyl ester (TAMe). Alkali was continuously added to keep the *p*H at 7.0 as the TAMe hydrolyzed. Esterase activity was there at zero time, and was there during the 30 min. of observation (FIGURE 5). The emphasis is now on the sequence of mixing the reagents and the time interval between each addition. In the second experiment thrombin was mixed with prothrombin and, as soon as possible, usually after less than 8 sec., the mixture was added to TAMe. The resulting mixture then contained prothrombin (38 units/ml.), thrombin (8 units/ml.), and 0.01 M TAMe. Alkali

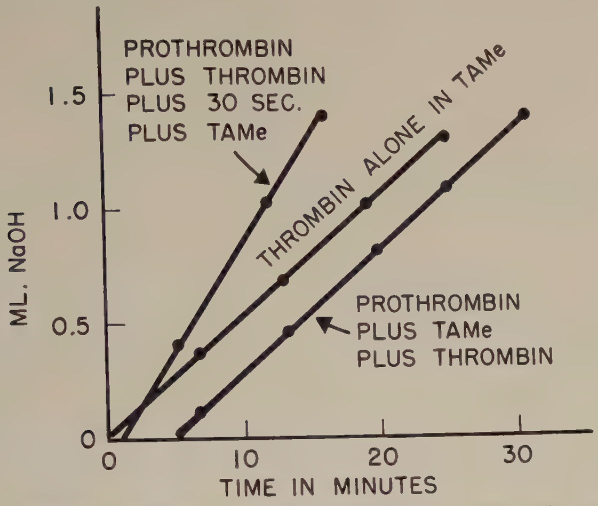

FIGURE 5. An enzyme substrate complex consisting of thrombin and prothrombin forms under suitable conditions.

was added to keep the *p*H at 7.0. There was no TAMe hydrolysis for 4 to 5 min., then hydrolysis proceeded at a rate equal to that observed when the prothrombin was not first mixed with the thrombin. In this experiment there was a period of 4 to 5 min. when thrombin was occupied with prothrombin and not with TAMe. TAMe freed thrombin from the prothrombin-thrombin complex and itself became hydrolyzed by the thrombin set free and in no way by additional or "new" thrombin molecules derived from prothrombin. The prothrombin, in some form perhaps not like the original, remained in the solution.

In experiment 3 we allow the mixture of prothrombin and thrombin to stand

TABLE 1

$$\text{Prothrombin} \xrightarrow{\text{Thrombin}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

Procoagulants	Anticoagulants
Calcium ions Tissue procoagulants Ac-globulin Platelet factor 3 Platelet cofactor I Prothrombin derivatives Lipid procoagulants Others	Calcium removal Heparin Sphingosine Inhibitors of (1) Tissue procoagulants (2) Platelet cofactor I (3) Platelet cofactor II (4) Ac-globulin Antithrombin Fibrinogen Others

for exactly 30 sec. before adding it to TAMe. The final mixture again contained prothrombin (38 units/ml.), thrombin (9 units/ml.), and TAMe (0.01 M). For approximately 1 min. no TAMe was hydrolyzed, but then hydrolysis started and proceeded at a rate much higher than in experiments 1 and 2. A thrombin-prothrombin complex formed, and new thrombin also entered into a complex to make the prothrombin-thrombin complex larger than in experiment 2. TAMe then freed the preformed thrombin plus the thrombin derived from the prothrombin, and this total quantity of thrombin entered into the hydrolysis of TAMe.

Thrombin is an activator of prothrombin. If I look for another enzyme as one of the clotting factors now discussed in the literature, which one would be an enzyme? Not one of them qualifies unequivocally as an enzyme. It might be possible eventually to see clearly that one or more qualifies for that classification. Thrombin alone suffices as the required enzyme; and being freed of the necessity for another one, I can see the function of other clotting factors as *aids to thrombin* when it exercises its qualities as an enzyme. If we then include those substances that *interfere with thrombin*, the whole perspective on prothrombin activation is simplified; since prothrombin is the center of blood coagulation, we can then go on to say that the perspective on blood coagulation is simplified. Two equations can state the basic events. Other factors support the production and enzymic function of thrombin, and these are called procoagulants. Opposed to these and, normally, almost in exact balance, are those factors that hinder the production or function of thrombin, and these are called anticoagulants. This is summarized in TABLE 1.

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THROMBIN AND ITS INTERACTION WITH FIBRINOGEN*

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Introduction

While the primary interest in thrombin has arisen heretofore because of its important role in the fibrinogen-fibrin conversion, this enzyme has recently received considerable attention because of its action on synthetic substrates and its possession of trypticlike properties.[1-10] In addition, considerable advance in the purification of thrombin has been made in the last two or three years. Rasmussen[11] has demonstrated that most of the active component of commercial thrombin preparations can be separated from the inactive protein impurities by chromatography on columns of Amberlite IRC-50 resin. This technique has been studied further by Seegers and Levine[12] and others, and it should provide the purest thrombin yet obtained. However, to date the enzyme has not been crystallized. With the availability of pure thrombin and its demonstrated action on a number of substrates, there is much work in progress at present on the structure of the enzyme and its mechanism of action.

From the preliminary information available at this time, the enzyme appears to contain not only protein but also a small amount of carbohydrate.[12] Hence, even though the prothrombin-thrombin conversion involves a liberation of carbohydrate,[13] some of the carbohydrate seems to be retained by the thrombin. The amino acid composition has been determined, as well as a portion of the amino acid sequence around the seryl residue to which di-isopropylfluorophosphate (DFP) is bound.[14]

Thrombin is inhibited by DFP,[2-4] but not by soybean trypsin inhibitor (STI).[15-17] Its activity is considerably decreased in the presence of formaldehyde, urea, sodium bromide, and a variety of other substances.[5, 6, 18, 19] The purified enzyme is quite stable in solution.[5, 12] Its activity can be destroyed by prolonged boiling in 6 M urea, although traces of thrombin activity were detected after a 10-minute boiling period.[20] Recently, Miller[21] has shown that all activity is lost in 8 M urea, but reappears if the urea concentration is reduced below 1 M.

Estimates of the molecular weight place it in the range of 15,000 to 20,000. These are based on measurements of the sedimentation constant (without parallel diffusion measurements),[12, 22] a minimum molecular weight from amino acid analysis,[9, 14] and the reaction of DFP with thrombin under the assumption that there is one mole of DFP bound per mole of thrombin.[9] It would be desirable to have a direct measurement of the molecular weight of the enzyme by one of the conventional techniques (for example, by sedimentation with the Archibald method[23]).

Thrombin catalyzes the hydrolysis of *p*-toluenesulfonyl-L-argine methyl

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* The preparation of this manuscript and the work of the author and his collaborators (M. Laskowski, Jr., S. Ehrenpreis, and T. H. Donnelly), to which reference is made here, were supported by Research Grant H-1662 from the National Heart Institute, Public Health Service, Bethesda, Md.

ester (TAMe),[1] lysine ethyl (and methyl) ester[8, 10] (LEe and LMe), the lysyl-alanine bond in the oxidized B chain of insulin,[8] and arginyl-glycine bonds in fibrinogen.[14, 24] It is not yet established[8] whether the arginyl-glycine bond in the oxidized B chain of insulin can be hydrolyzed by thrombin. The enzyme can also hydrolyze gelatin.[21] The optimum *p*H for hydrolysis of most of these substrates appears to be around 8;[21] the only exception appears to be the lysine ester, where the optimum *p*H is around 7.[10] The hydrolysis of LEe is not due to plasmin, since STI inhibits this enzyme, but fails to inhibit the hydrolysis of the lysine ester by thrombin.[8]

A comparison has been made of the Michaelis-Menten constants for thrombin and trypsin toward TAMe and LMe.[10] From the data thus far available, the activity of thrombin toward LMe appears to be significantly lower than toward TAMe. Because of this behavior it has been suggested[8] that, whereas the primary action of thrombin on fibrinogen is toward arginyl-glycine bonds,[14, 24] there may also be a much slower hydrolytic action toward lysyl bonds. Such a reaction might account[8] for the Guest-Ware effect.[15]

Interaction of Thrombin and Fibrinogen

Fibrinogen may be converted to a fibrin clot in the presence of thrombin. The reaction mechanism (in the absence of serum factor and calcium ions) may be formulated in terms of three reversible reactions:

$$\text{Proteolysis} \qquad F \overset{T}{\rightleftharpoons} f + P \qquad (1)$$

$$\text{Polymerization} \qquad nf \rightleftharpoons f_n \qquad (2)$$

$$\text{Clotting} \qquad mf_n \rightleftharpoons \text{fibrin} \qquad (3)$$

where F is fibrinogen, T is thrombin, f is fibrin monomer, P represents peptide and carbohydrate material, f_n represents intermediate polymers, and n and m are variable numbers. The work of a number of investigators who have provided the evidence for this reaction scheme has recently been reviewed.[20]

Suitable reagents are available[25] to inhibit one or more of these reactions so that the various equilibria can be individually investigated. For example, in 1 M NaBr at *p*H 5.3 steps 2 and 3 are inhibited; thus, step 1 can be studied.[26] Also, it is possible to dissolve fibrin in this same solvent to give fibrin monomer,[26] which is obtainable free of F, T and P. Step 2 can be investigated if the *p*H of the 1 M NaBr solution of f is raised to 6. If the *p*H is increased above 6, then step 3 occurs.

Step 1. This step has been extensively investigated[27-30] in 1M NaBr at *p*H 5.3 where steps 2 and 3 do not occur. The reason why steps 2 and 3 are inhibited in this solvent is discussed below under *Step 2.*

Before investigating the equilibrium of step 1 it was necessary to establish the reversibility[31, 32] of the reaction. As a criterion of reversibility, it was required that the same equilibrium position be reached from both the forward and reverse directions. The forward reaction was studied by mixing F and T in 1 M NaBr at *p*H 5.3, withdrawing aliquots at various times and analyzing for f. The reverse reaction was studied by mixing F and T in 1 M NaBr at

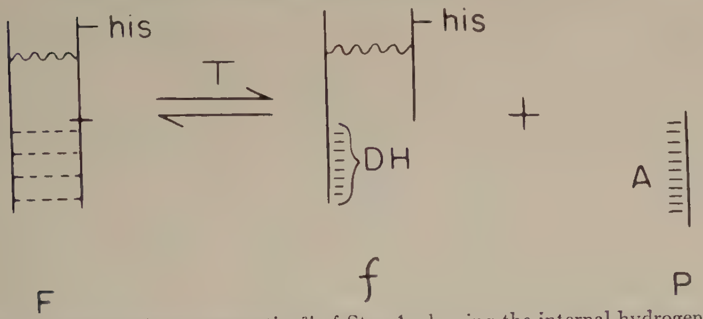

FIGURE 1. Schematic representation[34] of Step 1, showing the internal hydrogen bonds in *F* between donors (*DH*) and acceptors (*A*). The *DH* groups are probably tyrosyl residues,[35] and the *A* groups may be ionized carboxyl groups.[28] The histidyl groups are thought[26] to be present in *F* and to function as acceptor groups in Step 2 after the *DH* groups are liberated in Step 1.

*p*H 6; at this *p*H step 1 goes essentially to completion, since step 2 also occurs. The *p*H was then reduced to 5.3, reversing step 2 rapidly and completely; the slower reverse reaction of step 1 was then followed to equilibrium by similar analyses for *f*. In order to obtain correct values for the concentration of *f*, the thrombin in the aliquot taken for analysis must be inhibited. For this purpose, use was made of the competitive inhibition produced by TAMe;[1] that is, TAMe was added to the various aliquots to inhibit the thrombin* during the analysis for *f*. In this manner it was possible to obtain data on both the kinetics[19, 27, 29] and equilibrium[28, 30] of step 1.

The most extensive experiments were carried out at 0° C., at which temperature the same equilibrium position was attained in both the forward and reverse directions. Furthermore, the equilibrium position differed from that usually found for peptides of low molecular weight[33] in that step 1 does not go to completion under the experimental conditions used. A somewhat over-simplified explanation for this behavior[30, 34] can be presented in the terms of FIGURE 1. A more involved one has been provided by Laskowski *et al.* elsewhere.[30] The *F* molecule is represented schematically by two peptide chains connected by disulfide bonds (wavy line) and by hydrogen bonds between the side-chain polar *R* groups (dashed lines). In order to liberate *P*, not only must a peptide bond (short horizontal stroke) be split, but the side-chain hydrogen bonds connecting *P* to the remainder of the *F* molecule must also be broken. The additional free energy required to break the hydrogen bonds stabilizes the peptide bond against proteolysis.[34] If these hydrogen bonds did not exist, the hydrolysis of the peptide bond would proceed to completion. The quantitative aspects of such limited proteolysis have been presented elsewhere.[34]

This model accounts not only for the observed equilibrium position of the proteolytic reaction, but also for the need of a proteolytic step. Presumably, the role of thrombin in the fibrinogen-fibrin conversion is solely to liberate P and thus expose the side-chain donor groups DH required for the polymerization of step 2 (see below). Since P is acidic, the acceptor groups A, which mask the DH groups in the F molecule by internal hydrogen bonding, are presumably carboxylate ions of glutamic and aspartic acid side chains. As previously reported[28, 30] about 8 to 10 hydrogen bonds connect the f and P fragments in the F molecule.

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* More recently, Lorand and Yudkin[7] have shown a similar inhibitory effect of tosylarginylglycine on the clotting of fibrinogen. Presumably, this substrate acts in the same manner as TAMe. However, the rate of hydrolysis of tosylarginylglycine is slower than that of TAMe.[7]

Step 2. Once the f molecules are produced, they can polymerize at an appropriate pH to form the elongated polymers f_n. Again referring to FIGURE 1, the polymerization is thought to arise by formation of intermolecular hydrogen bonds between the donor groups DH liberated in step 1 and histidyl acceptors, which are assumed to be present even on the original F molecule.[26, 36, 37] Hydrogen bonds, rather than stronger covalent bonds, are thought to be involved here since the species f_n is readily depolymerized under relatively mild conditions.

The donors and the acceptors are involved in equilibria of the types

$$DH \rightleftharpoons H^+ + D^- \tag{4}$$

$$AH^+ \rightleftharpoons H^+ + A \tag{5}$$

$$DH + A \rightleftharpoons DH \cdots A \tag{6}$$

Formation of a hydrogen bond, according to EQUATION 6, between a given donor and acceptor disturbs the acid-base equilibria[36, 37] of EQUATIONS 4 and 5. Hence, at the low pH end of the range of polymerization, protons will be produced because of the polymerization-induced dissociation of ΔH^+, whereas protons will be taken up at the high pH end of the polymerization range when the polymerization cause D^- groups to combine with hydrogen ions.

Quantitative studies of the equilibria involved[36] indicate that there are 19 histidyl acceptors and 19 donor groups (probably tyrosyl residues[35, 36]) between which hydrogen bonds can form. The actual number of hydrogen bonds formed between these groups is pH-dependent, with a maximum number of about 10 being formed around neutral pH. The number of hydrogen bonds drops to zero around pH 5 (where the histidyl groups acquire protons and can no longer function as acceptors) and around pH 10.5 (where the tyrosyl groups lose their protons and can no longer function as donors). Thus, the model accounts for the pH range, about 5 to 10.5, in which polymerization occurs. For this reason, as already indicated, step 2 does not occur under the conditions described above for the study of step 1 (1 M NaBr, pH 5.3). The model also accounts for pH changes occurring during polymerization,[35, 36, 37] as well as for the observed pH-dependence of the heat of polymerization.[36]

Step 3. While a great deal is known of the morphology of fibrin clots,[38] little is yet known of the mechanism by which the intermediate polymers are organized into the fibrin network. There are various indications that fibrin may possibly be a crystalline polymer. For example, transitions have been observed in the X-ray diffraction patterns of fibrinogen and fibrin as a result of stretching and heating;[39,40] supercontraction on heating also has been observed.[39, 41] The possible role of crystallinity in fibrin formation is at present under investigation in this laboratory.[41]

Summary

Thrombin, like trypsin, has hydrolytic activity toward arginyl and lysyl ester and peptide bonds in several synthetic and protein substrates. In fibrinogen, the action of thrombin appears to be specific for two arginylglycine bonds to liberate peptide material and "expose" the donor groups required for the subsequent polymerization. Thrombin does not take part in the polymerization steps. Hydrogen bonding between side-chain polar groups plays an important role in the various equilibria in the fibrinogen-fibrin conversion.

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Discussion of the Paper

Walter Troll (*Institute of Industrial Medicine, New York University-Bellevue Medical Center, New York, N. Y.*): It is interesting to note that TAMe is being used as an immediate thrombin inhibitor in the unraveling of the fibrinogen-fibrin conversion. I want to record, however, a minor point of disagreement, which is also based on the use of synthetic esters as inhibitors of thrombin. I am referring to your report that thrombin is also able to split lysine ethyl ester (LEe). My colleague Sol Sherry and I have observed that LEe, even in high concentrations, does not interfere with the clotting action of thrombin on fibrinogen. We wonder if the purity of the thrombin was sufficient to exclude the possibility of a LEe esterase impurity.

Harold A. Scheraga. If the concentration of LEe in the experiments of Sherry and Troll was high enough to attain the zero order region in LEe and, further, if the LEe esterase site in the thrombin molecule is *assumed* to be the same as the fibrinogen clotting site, then Walter Troll is correct in believing that the LEe esterase activity could not be due to the thrombin itself, but rather to an impurity in it. This is due to the fact that the thrombin-fibrinogen reaction is first order in substrate, and the same enzyme cannot simultaneously catalyze a zero-order reaction on one substrate and a first-order reaction on a second substrate.[1] However, it is not known whether both sites on thrombin are identical. If they are *assumed* to differ, then thrombin could simultaneously catalyze a zero-order hydrolysis of LEe and a first-order hydrolysis of fibrinogen; that is, LEe would not interfere with the thrombin-fibrinogen reaction. In other words, LEe could completely cover up one active site on thrombin, leaving the other site to react with fibrinogen. Acid denaturation, which destroys both activities of thrombin, could affect both sites.

If our assumption of two different sites is incorrect, then the LEe esterase activity would be due to an impurity in thrombin. If such were the case, the impurity could not be plasmin or trypsin, since both of these enzymes are inhibited by soybean trypsin inhibitor (STI), whereas thrombin is not. We have found that STI does not inhibit the action of thrombin on LEe or on the lysyl-alanine bond of the B chain of oxidized insulin.

In conclusion, the LEe esterase activity is probably not due to plasmin or trypsin. It may be due to some other proteolytic impurity in thrombin or to a site on the thrombin molecule that is not involved in the thrombin-fibrinogen reaction.

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PHOSPHATIDES AS PLATELET SUBSTITUTES IN BLOOD COAGULATION*

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In recent years it has become apparent that the platelets play a complex role in the hemostatic mechanism, and a number of specific functions are now recognized. These are summarized in TABLE 1. In the coagulation of the blood, factors contained in platelets accelerate the conversion of prothrombin to thrombin (Factor 1),[1] accelerate conversion of fibrinogen to fibrin (Factor 2),[1] participate in the formation of thromboplastin (Factor 3),[2] and neutralize the action of heparin (Factor 4).[3, 4] Not involved in coagulation, but significant in hemostasis, are the functions of platelets in promoting clot retraction[5] and vasoconstriction.[6]

In 1936 Chargaff *et al.*[7] demonstrated an accelerator of coagulation in lipid extracts of platelets, and in 1952 Van Creveld and Paulssen[2] demonstrated that the platelet factor participating in thromboplastin formation (Factor 3) is a phospholipid. Since then numerous phosphatides of plant and animal origin have successfully been substituted for platelets in a variety of test systems designed to measure thromboplastin formation.[8-10] The active phospholipids have uniformly been cephalins, and most studies have indicated that the active principle is probably the ethanolamine phosphatide.[11]

Of the four factors participating in coagulation, Factor 1 is clearly a protein[1] and Factor 3 a phospholipid.[2] However, the factors that accelerate the thrombin-fibrinogen reaction (Factor 2) and which neutralize heparin (Factor 4) have been found in water-soluble platelet fractions by some workers[1, 4, 12] and in insoluble and lipid fractions by others.[2, 13]

The studies reported here concern the properties of a phospholipid derived from soybeans. In this study the phosphatide was tested for each of the known functions of platelets. The aims of the investigation were twofold: first, to delineate, by *in vitro* testing, those functions that could be attributed to the phospholipid content of platelets; and, second, to evaluate, by *in vivo* testing, the relative importance of the various platelet functions in the actual control of thrombocytopenic bleeding.

Since platelet Factor 3 had been shown to be a phosphatide, soybean phospholipids were initially tested in the thromboplastin generation test.[14] FIGURE 1 demonstrates the comparative activity of the crude phosphatide, the alcohol-soluble (lecithin) fraction, and the alcohol-insoluble (cephalin) fraction.† Over a wide range of concentration activity could be demonstrated with crude phosphatide and in the cephalin fraction, while the lecithin fraction was virtually inactive. Comparative studies of the crude phosphatide and the cephalin fraction demonstrated that the greater activity, per unit weight, was

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† Supplied by Joseph Eichberg, Associated Concentrates, Woodside, N. Y.

TABLE 1

FUNCTIONS OF THE BLOOD PLATELETS

In blood coagulation:
- Accelerate conversion of prothrombin to thrombin (Factor 1)
- Accelerate conversion of fibrinogen to fibrin (Factor 2)
- Participate in formation of thromboplastin (Factor 3)
- Neutralize heparin (Factor 4)

In hemostasis:
- Promote clot retraction
- Promote vasoconstriction (serotonin).

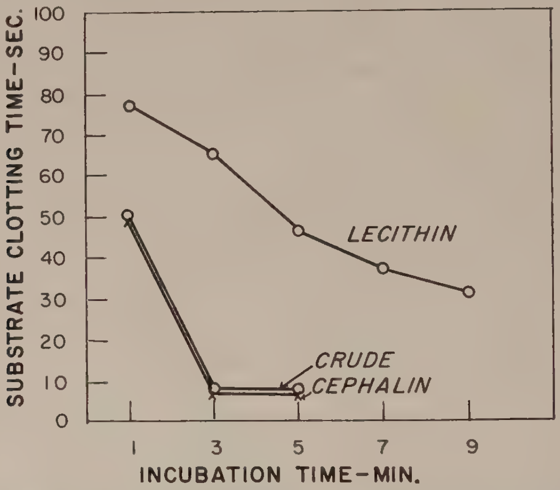

FIGURE 1. Coagulant activity of soybean phosphatide fractions. Comparative activity of crude soybean phosphatide, the alcohol-soluble (lecithin) fraction, and the alcohol-insoluble (cephalin) fraction in the thromboplastin generation test. Test system: 0.3 ml. normal adsorbed plasma (1:5), 0.3 ml. lipid emulsion (0.025 gm. per cent), 0.3 ml. normal serum (1:10), 0.3 ml. $CaCl_2$ (.025 M); substrate, normal plasma.

in the cepahlin (FIGURE 2) and this was then chosen for all further studies. This material contains approximately two thirds inositol phosphatide, one third ethanolamine phosphatide, and about 4 per cent choline phosphatide.

FIGURE 3 demonstrates a comparison between platelets and soybean cephalin in the thromboplastin-generation test. It may be seen that thromboplastin generation equal to that obtained with platelets (200,000 to 600,000/cu. mm.) is achieved with lipid concentrations ranging from 12 to 50 mg./100 ml. of saline. At higher concentrations there is a progressive decrease in the rate of reaction, with an eventual reduction in yield as well. At a lipid concentration of 6 mg. per cent the reaction rate is very rapid, but a diminished yield of thromboplastin is again seen.

The comparative activity of platelets and soybean cephalin in correcting the abnormal prothrombin consumption of thrombopenic plasma is seen in FIGURE 4. Increasing concentrations of the lipid produce progressive improvement in prothrombin consumption. With a final lipid concentration of

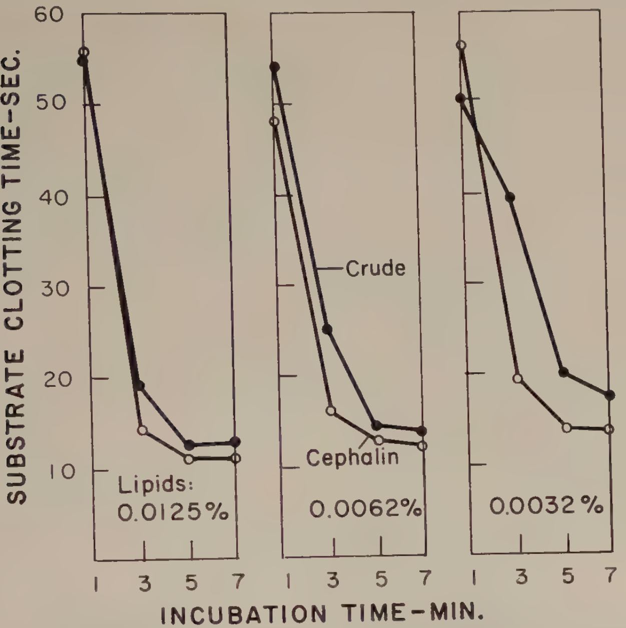

FIGURE 2. Comparative activity of the crude soybean phosphatide and the alcohol-insoluble (cephalin) fraction on thromboplastin generation at various concentrations. Open-circle line, crude soybean phosphatide; solid-circle line, cephalin fraction.

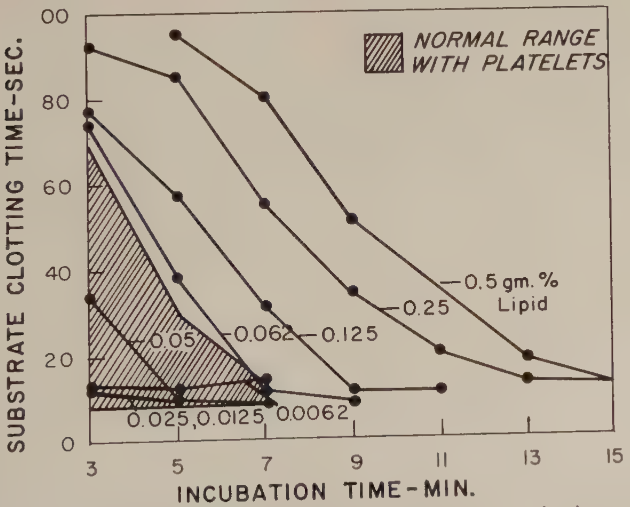

FIGURE 3. The effect of phosphatide concentration on thromboplastin generation. Incubation mixture: 0.3 normal adsorbed plasma (1:5), 0.3 ml. normal serum (1:10), 0.3 ml. $CaCl_2$ (0.025 M), 0.3 ml. lipid emulsion; substrate, normal whole plasma.

10 to 20 mg./100 ml. of plasma, the correction is equal to that obtained with 250,000 platelets per cu.mm. of plasma. At lipid concentrations over 50 mg. per cent inhibition begins to become apparent.

FIGURE 5 demonstrates the corrective effect of cephalin on the prothrombin

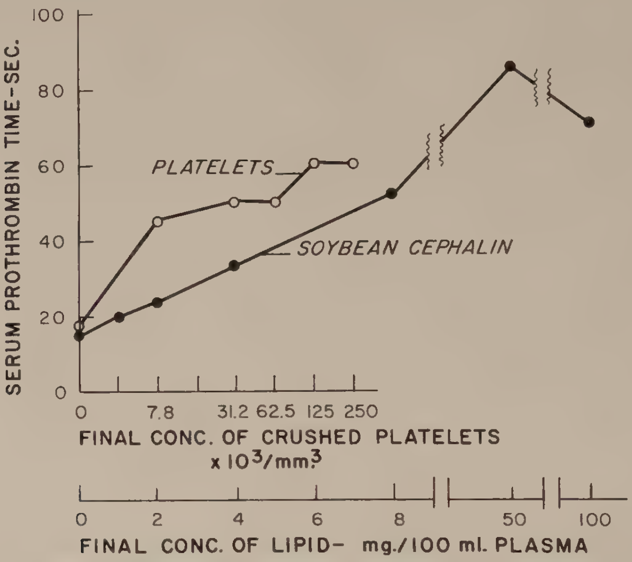

FIGURE 4. Effect of platelets and soybean cephalin on prothrombin consumption of thrombocytopenic (leukemic) plasma.

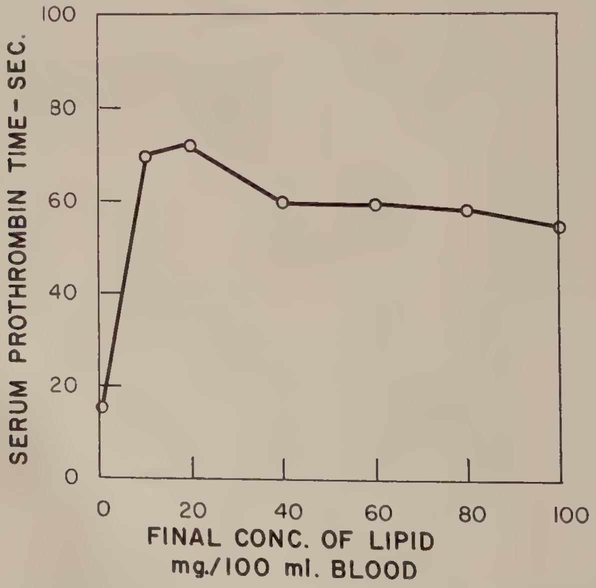

FIGURE 5. The effect of soybean cephalin on prothrombin consumption of thrombocytopenic (lupus) whole blood.

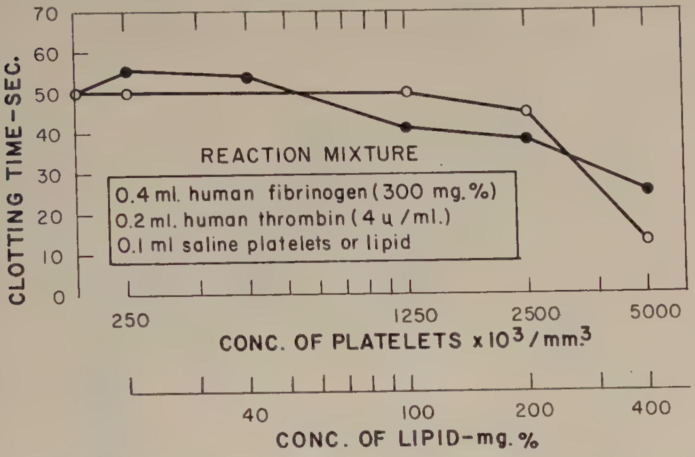

FIGURE 6. The effects of platelets and soybean cephalin on the thrombin-fibrinogen reaction.

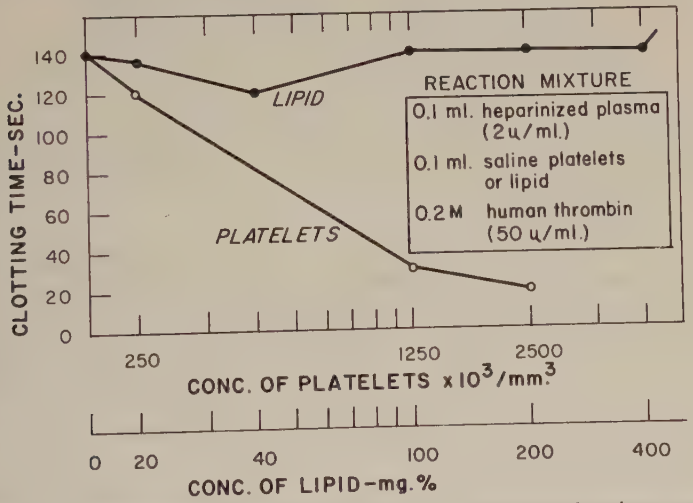

FIGURE 7. Comparison of the antiheparin effects of platelets and soybean cephalin.

consumption of thrombocytopenic whole blood. Again, a final concentration of 20 mg./100 ml. of blood produces the maximum correction.

FIGURE 6 demonstrates the comparative activity of platelets and cephalin in the thrombin-fibrinogen reaction. It may be noted that both materials accelerate the reaction. Relatively high concentrations are required in both systems, the lipid being somewhat more active at lower levels.

As indicated in FIGURE 7 no antiheparin effect of the cephalin could be demonstrated, whereas a marked antiheparin effect of platelets is evident. This study confirms the observations of Deutsch *et al.*[4] that the thromboplastic and antiheparin factors of platelets are distinct.

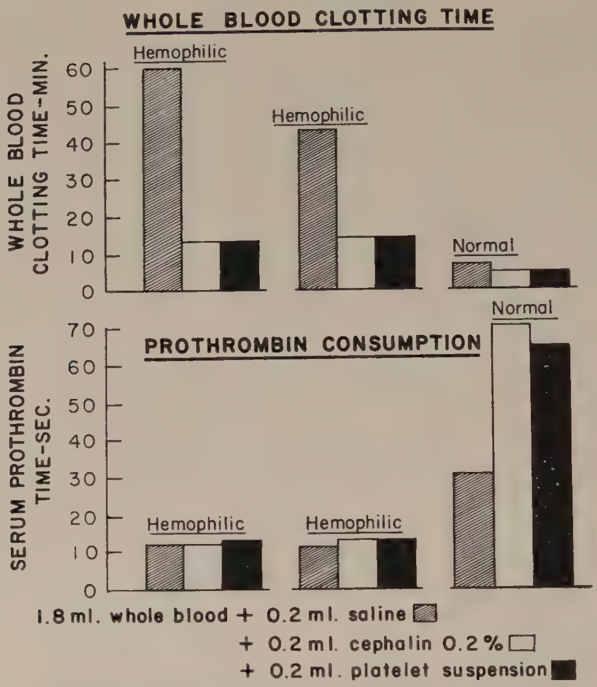

FIGURE 8. The effects of soybean cephalin and platelets on whole blood clotting time and prothrombin consumption of hemophilic blood.

An interesting property of the lipid was found in its effect on hemophilic blood (FIGURE 8). Both platelets and the soybean cephalin reduced the prolonged clotting time of hemophilic blood to normal. With neither preparation, however, was any improvement in prothrombin consumption noted. It would appear that this property of platelets and cephalin has little bearing on the fundamental abnormality in hemophilia and probably represents an expression of their effects on the thrombin-fibrinogen reaction.

It was apparent in studies with the lipid that physical state, as well as concentration, played a role in the activity. This is shown in FIGURE 9. At all concentrations activity could be improved by homogenizing the emulsion and reducing the particle size; this was most evident at high concentrations.

As demonstrated thus far, the soybean cephalin possessed thromboplastic and fibrinoplastic activity. It was found to have neither activity in accelerating conversion of prothrombin to thrombin nor antiheparin effect. Additional studies demonstrated that the cephalin possessed no vasoconstrictor activity and caused only minimal improvement in clot retraction. A summary of the properties of the soybean cephalin as compared with platelets is shown in TABLE 2.

Despite the fact that the soybean lipid possessed only two of the six known platelet functions use of the material *in vivo* indicated that it was capable of improving thrombocytopenic bleeding in humans. In preliminary animal experiments, 50 ml. of a 2 per cent emulsion was injected into rabbits (300

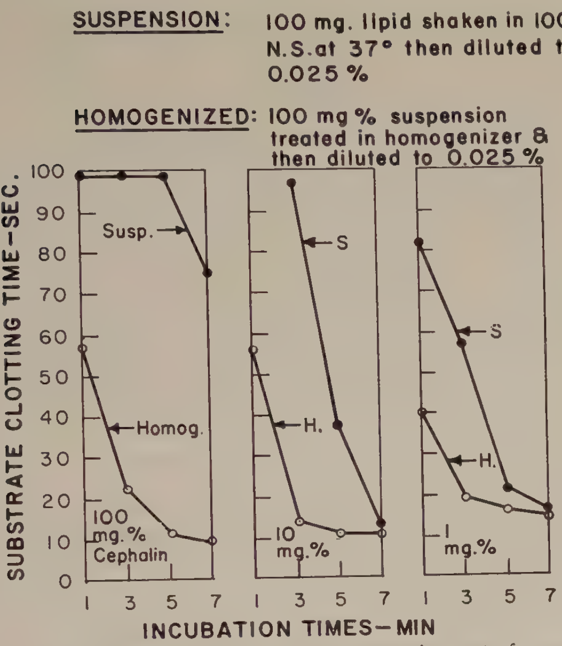

Figure 9. The effect on the thromboplastin generation test of varying the particle size of the soybean cephalin. Suspension: 100 mg. lipid, shaken in 100 ml. normal saline at 37° C., then diluted to 0.025 per cent. Homogentate: 100 mg. per cent solution treated in homogenizer and diluted to 0.025 per cent.

Table 2

Comparison of Functions of Platelets and Soybean Cephalin

Platelets	Soybean cephalin
Prothrombin → thrombin acceleration	0
Fibrinogen → fibrin acceleration	+
Thromboplastic factor	+
Antiheparin activity	0
Clot retraction	0
Vasoconstrictor activity	0

mg. cephalin/kg. of body weight). No immediate toxicity was evident; neither was any accumulation of lipid noted in the tissues on post-mortem examination after sacrificing the animals 5 days later. The massive doses did not produce hypercoagulability of the blood in the rabbits but, as might have been expected from the *in vitro* experiments, they exerted an anticoagulant effect. The lipid was cleared from the blood in 6 hours, by which time the coagulation status had returned to normal. In humans the cephalin was administered intravenously as a 0.5 per cent emulsion in 5 per cent dextrose water. An initial dose of 10 mg. per kg. of body weight was followed by a slow drip that supplied 60 mg./kg./24 hours. The patients tested thus far have been children critically ill with far-advanced leukemia. In these patients the hemorrhagic manifestations were so gross that any benefit would be un-equivocal. The patients selected presented severe epistaxis, hematuria, or melena, which had persisted for at least 24 hours despite the administration of transfusions of whole blood. All of the patients had been on corticosteroids prior to the onset of bleeding. Clear-cut benefit was evident in 4 of the 7 children studied, while the remaining 3 showed no improvement. In the patients who responded to the administration of cephalin, evidence of diminution of bleeding was noted within 3 to 8 hours following onset of therapy.

In the preliminary clinical studies only acute cessation of bleeding was tested. Factors of dosage, frequency of administration, and effectiveness in thrombocytopenic states of varied etiology are now under study. The results thus far suggest, however, that not all of the platelet functions are necessarily required for control of thrombocytopenic hemorrhage. The observations are in accord with those of Hayhoe and Whitby,[15] who noted improvement in thrombocytopenic bleeding following intravenous administration of a chloroform extract of human brain in 10 leukemic adults.

Summary

A soybean cephalin has been found to demonstrate thromboplastic and fibrinoplastic effects similar to those of platelets. Despite the absence of 4 of the 6 known platelet functions, the lipid holds promise in the therapy of thrombocytopenic states.

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A SURVEY OF SOME PLATELET ENZYMES AND FUNCTIONS: THE PLATELETS AS THE SOURCE OF NORMAL SERUM ACID GLYCEROPHOSPHATASE*

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Although one goal of biochemistry is to relate chemical activity to cellular function, many biochemical studies must of necessity be carried out under conditions that are highly unphysiological. When we deal with homogenates, separated cell particles, and even tissues slices, we are dealing with cells and cell fragments that are no longer in their normal environment and relationship. Blood represents a unique tissue because its matrix is fluid. Consequently, there is a more reasonable possibility of studying its cells with a minimum of mechanical trauma and of investigating the effects of mechanical trauma itself. In blood, it is not only easier to make chemical measurements under reasonably normal conditions, but it is also possible to measure some aspects of function *in vitro* under conditions not too far removed from those prevailing in the body. Compared to solid tissues, blood may afford better opportunities to correlate biochemical findings with physiological functions.

In this review, we shall first consider some of the physiological aspects of the blood platelets and attempt to correlate them with biochemical phenomena. Then the literature on platelet enzymes will be reviewed and, finally, some new data on certain enzymes will be presented. For reference to the literature up to 1954, Maupin's book on platelets[1] is most helpful.

Biochemical data support the morphologic and physiological evidence that the platelets are fragments of the cytoplasm of megakaryocytes, since both platelets and megakaryocyte cytoplasm stain identically for enzymes[2-4] and for nonenzymatic constituents such as nucleic acids,[2,5] glycogen,[6-9] and lipid.[10a,10b] The platelets, therefore, are of interest as samples of cytoplasm presented to us by nature. As one might expect, they contain no demonstrable desoxyribonucleic acid, but do have small amounts of ribonucleic acid.[1,2a,2b,5,11,12] Greene believes that the major component of platelets is ribonucleoprotein.[13] In addition, a considerable amount of adenosine triphosphate (ATP) is present.[12,14-16] No specific data on di- or triphosphopyridine nucleotide were found. A sulfated mucopolysaccharide is present.[17] The effect of hyaluronidase and of lysozyme on staining characteristics[2b,18a-18c] is disputed. Platelets contain between 0.31[1] and 0.39[12] mg. N per 10^9 platelets,† of which 83 to 92 per cent is protein nitrogen.[12] Tryptophan,[12] tyrosine,[12] and other[15] amino acids in the protein have been measured. Phosphorus partition[1,11,12,14] and

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† According to Maupin's figures on human platelets,[1] the dry weight of platelets is 12.5 per cent of the wet weight; 10^9 platelets weigh 2.8 mg. dry weight; 11 per cent of the dry weight is nitrogen. Fantl and Ward[12] found 5×10^{10} platelets per gm. wet weight. Other useful figures are: by volume 1 red blood cell equals 8 platelets, and 1 white blood cell equals 90 platelets.[12] Platelet volume in man has been estimated at 7.3 μ^3,[19] and specific gravity as 1.03.[20]

nucleotide composition[12] have been studied. Microsomes and vacuoles are present[21-24] and at least some platelets contain mitochondria.[21, 22, 24] Platelets are rich in phospholipid.[24a, 25] Calcium, Mg, Cu, Fe, and Mn are present; Zn is absent.[1] Carotenoid pigments occur in platelets.[25a]

Platelets in the circulating blood are in the shape of flat disks, more or less coin-shaped.[21, 26] Maintenance of this shape seems to depend upon the ionic environment, the presence of colloids, and the temperature.[21, 27] To some extent, changes in shape are reversible. The disk shape is retained for at least an hour if blood is collected in sodium citrate or oxalate and kept at 37° C. If the blood is chilled, the platelets become spheres, usually with protruding filaments, but many of them again resume the disk shape when the blood is rewarmed.[27] Magnesium in minute concentration is important in maintaining the disk shape; if disodium ethylenediaminetetraacetate (EDTA) is used as anticoagulant in a concentration sufficient to chelate all of the plasma magnesium, the platelets are mostly spherical. If less sodium EDTA is used, or if magnesium EDTA is employed as anticoagulant, or if a very slight excess of magnesium is added to blood collected with the usual concentration of sodium EDTA, most of the platelets are seen to be disk-shaped.[28] Colloids, too, seem to play a role in the maintenance of the disk form. Platelets suspended in saline are spherical, even when kept at 37° C., but if albumin or gum acacia is added, many of them revert to disks.[27] The more specific biochemical implications of these ion, temperature, and colloid effects upon platelet shape are not yet known.

Estimates of the life span of human platelets vary between five[29] and nine[30, 31] days. Virtually nothing is known about the physiology or biochemistry of the senescence or the fate of the circulating platelets.

The major function of the platelets is to produce hemostasis. This can be divided into three aspects: first, clumping and fusing of the platelets at the site of the blood vessel injury; second, the function of the platelets in blood clotting; and, third, the role of the platelets in maintaining the resistance of the capillaries to red cell extravasation.

With regard to the first of these aspects, the processes of agglutination and fusion of the platelets that produce hemostatic platelet plugs *in vivo* are presumably the same as those causing viscous metamorphosis[32] and clot retraction *in vitro*. Lüscher,[33] Bounameaux,[34] and Borrelli and I[35] have found that thrombin will not produce viscous metamorphosis of washed platelets when the platelets are suspended in isotonic saline, but will do so provided some other factor is present. Work in my laboratory has shown that this other factor is simply the maintenance of the *p*H near 7.4; if washed platelets are suspended in plasma dialyzate[33] or in imidazole, phosphate, veronal, or Tris buffer at *p*H 7.4,[35] the platelets do clump and fuse together when thrombin is added.

There is a great deal of evidence that viscous metamorphosis produces clot retraction.[36] Fibrin needles adhere to the platelets, and the clot is pulled together as the platelets agglutinate and fuse. However, there is evidence from some laboratories that not only fibrinogen, platelets, and thrombin, but also a nondialyzable serum factor is required for retraction.[37, 38] Bounameaux[34] has also reported other differences between viscous metamorphosis and clot retraction.

Almost all investigators agree that intact platelets are required for clot retraction and viscous metamorphosis. Frozen or homogenized platelets are ineffective.[36] There is no evidence that the platelets contain an enzyme, retractozyme, that is responsible for retraction.[36, 39] Calcium or magnesium in very low concentration is required.[28, 34] Many types of enzyme inhibitors prevent retraction;[34, 38, 40] their diversity suggests that many of the biochemical processes in the platelet must be functioning. Thus, to produce viscous metamorphosis and clot retraction, thrombin, an enzyme, must act upon the integrated platelet.

Of the role of platelets in coagulation I shall say very little. Although platelets can influence a number of the reactions of clotting through several different substances, they do not play an important enzymatic role. Rather, the essential substance that they contribute seems to be phospholipid.[41] Born[42] has recently found that ATP disappeared from the platelets during clotting and was not found in the clot or serum. Furthermore, protein-bound phosphorus and nitrogen increased when plasma was clotted in the presence of platelets.[43] This may reflect the formation of "plasma thromboplastin," although this protein does not contain phosphorus.[44] However, since thromboplastin generation is normal when phospholipid is substituted for platelets,[41] it seems probable that the enzymatic and protein components of the reaction are contributed by the plasma rather than by the platelets.

Finally, the role of the platelets in maintaining the integrity of the capillaries is poorly understood. Recent evidence indicates that injection of lyophilized platelets improves the capillary resistance of thrombocytopenic recipients.[45] This suggests that some chemical component of the platelets, perhaps a sulfated mucopolysaccharide,[17] is responsible, rather than viable circulating platelets.

I shall briefly mention another platelet function: that of binding various substances and thus transporting them in the blood. This carrier function is clearly established for serotonin (5-hydroxytryptamine), which in the circulating blood is bound to the platelets.[46, 47] There is good evidence that the serotonin in normal platelets has been taken up, probably from the gastrointestinal tract.[48] Platelets *in vitro* can take up serotonin when it is added to the plasma in which they are suspended,[49–52] although platelets *in vivo* can do so more effectively.[51] Washed human platelets cannot take up serotonin[52] unless phosphate or citrate ion is added[51] and the temperature is 37° C.[50] Platelet serotonin can be released slowly by the drug reserpine.[53] It is liberated rapidly by thrombin,[54] hence serum but not plasma is rich in serotonin. Local vasoconstriction produced by serotonin liberated from the hemostatic platelet plug may aid in arresting bleeding,[55] but is not essential.[53]

Platelets can also bind histamine, epinephrine, and norepinephrine[51, 56] although this has not been shown to play any role under physiological conditions. Protein molecules, too, can apparently be bound. There is good evidence that the substance in platelets that behaves like accelerator globulin or factor V is actually factor V taken up from the plasma.[57] Repeated washings do not remove it from the platelets. Furthermore, platelets bind fluorescein-labeled antibody to human gamma-globulin, even after they have been washed six times, which suggests that they may take up gamma-globulin, as well.[58] Finally, thoroughly washed platelets contain fibrinogen.[59, 60] This protein-binding ability of the platelets raises the question whether some of the enzymes found in platelets may have been taken up from the plasma or other tissues.

Let us turn now to a brief survey of the literature on platelet enzymes. It should be evident from the foregoing discussion that measurement of enzymatic processes in the platelets has many difficulties, even in this convenient tissue, blood. Since the platelets are profoundly altered by thrombin, clotting must be prevented. Campbell has found that different anticoagulants affect oxygen consumption very differently;[61] we have found differences with regard to platelet morphology[27] and ability to undergo viscous metamorphosis and produce clot retraction.[28] Thus the choice of anticoagulant is an important consideration. According to Campbell,[61] but not according to others,[1, 62, 63] washing the platelets abolishes their oxygen consumption. It also destroys their ability to survive after transfusion.[29] However, washing may be essential for studying platelet enzymes, especially those that occur in the plasma as well as in the platelets. Finally, histochemical findings on platelets must be viewed with the knowledge that the platelets, when spread out upon glass slides, separate into hyalomere and chromomere: a separation not present in the disklike circulating platelet.

TABLE 1 lists the enzymes that have been shown to occur in platelets.

Relatively few studies of physiological and pathological variations in platelet constituents have been made. Glycogen increases as megakaryocytes mature,[94] and megakaryocyte[94] and platelet[95] glycogen are low in thrombocytopenia. After two or three days' storage of whole blood, platelets lose their glycogen and alkaline phosphatase.[85c]

Salvidio and his co-workers have found a reduction in platelet 5-nucleotidase and alkaline phosphatase, and an increase in peptidase during menstruation.[81] In patients with hepatic cirrhosis, platelet nucleotidase, ATP-ase, acid, and alkaline phosphatase decrease; in obstructive jaundice with disturbed liver function, changes in these enzymes are not seen.[82] During the administration of dicumarol these enzymes, as well as peptidase, decrease, and are not rapidly restored by vitamin K_1.[96] Human platelet histaminase increases in pregnancy.[90]

Using platelets and serum prepared in our laboratory, Wróblewski's laboratory has measured the concentration of several enzymes with added pyridoxal.[97] Platelets were separated from normal human blood (1 vol. disodium ethylenediaminetetraacetate to 9 vol. blood) with siliconed glassware. They were washed 3 times with saline[98] and suspended in saline in a concentration of between 0.135 and 4.2 × 10^6 platelets per cu. mm. The presence in the platelets of glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and glutathione reductase (GR) was variable (TABLE 2). A considerable quantity of lactic dehydrogenase (LD) was always present, especially in samples left overnight at 4° C. or −20° C. (TABLE 2). No greater

TABLE 1
PLATELET ENZYMES

Enzyme	Present		Absent	
	Histochemical Methods	Other Methods	Histochemical Methods	Other Methods
Cytochrome oxidase		64*		
Succinic dehydrogenase	65*	66*		
Lactic dehydrogenase		66,* 67,* 67a		
Other dehydrogenases		66,* 67a		
Catalase		68, 69, 70*		
Peroxidase		71	2b,* 72	
Dopa-oxidase		73	72	
Oxidase		61,* 74	72	
Proteolytic (ovalbumin)**		76*		75
Trypsin-kinase		77		
Chymotrypsin			78*	
Carboxypeptidase			78*	
Peptidase†		79, 80, 81,* 96*		
Amylase		76*		
Lipase			78*	75, 76*
β-Glucuronidase	78*	83a,* 83b		
Acid phosphatase	78*	81,* 82,* 83b, 84, 85	85a*	
Alkaline phosphatase	85a,* 85b*	81,* 82*	2a, 2b, 78*	83b, 84, 85
5-Nucleotidase		81,* 86,* 82*		
ATP-ase		82*		
Pyrophosphatase		87,* 88		
Acetylcholinesterase	4‡	3,‡ 83b	78,* 4*‡	3*‡
Esterase	78*	83b		
Sulfatase		83b	78*	
Carbonic anhydrase		89*		
Histaminase		90*		
Histidine decarboxylase		91§		91§
Glutamic oxalacetic transaminase		92,* 67*		
Glutamic pyruvic transaminase		67*		
Glutathione reductase		67*		
Ribonuclease				93*
Lecithinase		1* (weak)		
Desaturase				1*

* Tested on human platelets.
** Platelets also have antifibrinolytic activity.[76a, 76b]
† Active against glycyl-tyrosine, glycl-glycine, alanyl-glycine and leucyl-glycine.
‡ Present in many mammals, absent in human platelets.
§ Present only in rabbit platelets, which contain much more histamine than the platelets of other mammals.

activity was seen in one experiment on unwashed platelets that were centrifuged out of the plasma and resuspended in saline, except for GOT, which was 150.

The platelet concentration of acid phosphatase, measured in Bodansky's laboratory by hydrolysis of β-glycerophosphate, and of LD was adequate to account for all of the activity of these enzymes in normal serum obtained from clotted whole blood (TABLE 3). It seemed possible, therefore, that the normal concentration of these enzymes in serum resulted from their liberation from

TABLE 2
ACTIVITY OF SOME ENZYMES IN PLATELETS[67]

Enzyme	Units/min./10^9 platelets		
	Fresh intact	4° C. overnight	−20° C. overnight
GOT	0–74	0–91	40 (1 expt.)
GPT	0–156	0–12	0–7
GR	Questionable activity	—	12, 37
LD	230–1020	630–1715	890–1270

TABLE 3
LACTIC DEHYDROGENASE AND ACID GLYCEROPHOSPHATASE ACTIVITY IN SERUM AND PLATELETS

Enzyme	Units	Serum normal values	Platelets washed, frozen 430,000/cu. mm.
Lactic dehydrogenase	Units/ml./min.	100 to 600	460 to 737
Acid phosphatase	μg. P/100 ml./hr.	0 to 800	146 to 778

the platelets during coagulation. To test this, we prepared serum from both platelet-rich and platelet-poor plasmas in six normal adult subjects. No anticoagulant was used; the blood was collected in chilled siliconed glassware. It was immediately centrifuged slowly to obtain plasma rich in platelets, but poor in red and white cells. One half of this plasma was then centrifuged rapidly in the cold to obtain platelet-poor plasma. The plasmas were placed in glass tubes at 37° C. for 1 hour while clotting occurred. One hour later the clots with the entrapped platelet residues were removed.

There was a striking and reproducible difference in the behavior of the enzymes (TABLE 4). Despite the presence of LD in the platelets, the concentration of the enzyme was the same in serum prepared from platelet-rich and platelet-poor plasmas. This was also true for GOT, GPT, GR, and alkaline phosphatase. In the case of acid phosphatase, however, no significant activity was observed in any sample of serum from platelet-poor plasma,* whereas the serum from platelet-rich plasma had definite activity in every experiment. Thus, serum acid glycerophosphatase activity in the normal individual appears to arise from the platelets.†

The question of why acid phosphatase is liberated from the platelets during coagulation, whereas LD is not so liberated is an interesting one, and it is currently under investigation. The simplest explanation would be that LD is bound to the platelet ghosts, which are removed with the clot, whereas acid phosphatase is soluble. However, when platelets are frozen, or suspended in distilled water, and then centrifuged vigorously to sediment the ghosts,

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* This lack of activity was not attributable to destruction of the enzyme at 37° C., since samples clotted at room temperature were also devoid of activity.

† Subsequent studies have shown that serum acid nitrophenylphosphatase activity is only partially derived from the platelets.

TABLE 4

LACTIC DEHYDROGENASE AND ACID GLYCEROPHOSPHATASE ACTIVITY IN SERUM FROM PLATELET-RICH AND PLATELET-POOR PLASMA

	Serum from	
	Platelet-rich plasma	Platelet-poor plasma
Lactic dehydrogenase	186	251
Units/ml./min.	(100–320)	(112–640)
Acid glycerophosphatase	320	0
µg. P/100 ml./hr.	(130–490)	(70–neg.)

most of the LD activity was found in the supernatant. Further work on this question is in progress.

The observation that acid phosphatase is normally present in serum because it is liberated from the platelets during clotting is of potential value in the clinical assessment of borderline elevations of this enzyme in patients with carcinoma of the prostate. To date we have studied acid phosphatase in only two patients with carcinoma of the prostate. Serum from platelet-poor plasma allowed to clot at 37° C. for an hour contained 6.33 and 0.35 Bodansky units (or 6330 and 350 µg. P/hour/100 ml.) respectively.* This is in contrast to the absence of any activity in similar serum obtained from normal individuals. The serum acid phosphatase in the second of these patients ranged between 0.7 and 0.9 Bodansky units, which is at the upper limit of the normal range.[99] Thus it appears that determination of acid phosphatase in serum from platelet-poor plasma is a more useful clinical test than measurement of acid phosphatase in the usual serum from clotted whole blood. Ways are being sought to make this test feasible on a routine basis.

Summary

The literature on platelet functions and on enzymes and other chemical constituents of platelets is reviewed. Platelets contain sufficient lactic dehydrogenase and acid glycerophosphatase activity to account for all of the activity in normal serum. However, serum lactic dehydrogenase is the same whether clotting does or does not occur in the presence of platelets. In contrast, serum activity against glycerophosphate, at acid *p*H is absent in serum obtained from plasma poor in platelets, but is normal when the serum is obtained from plasma containing a normal number of platelets. Thus, normal serum acid glycerophosphatase appears to come from the blood platelets. In contrast to normal individuals, acid glycerophosphatase activity was found in serum from platelet-poor plasma obtained from two patients with metastatic carcinoma of the prostate, suggesting that a more sensitive test for pathological elevation of acid phosphatase in prostate carcinoma is provided when the contribution of acid phosphatase from the platelets is avoided.

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* Some prostatic acid glycerophosphatase activity is destroyed at this temperature, since the concentrations were 9.79 and 0.80 respectively when the samples were left at 23° C. instead of at 37° C.

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Part IV. Serum Enzymes and Their Origin

ANALYTICAL CONSIDERATIONS IN SERUM ENZYME DETERMINATIONS

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The determination of the activities of some serum enzymes has long been indispensable to clinical medicine. Recently, the number of such useful enzymes has begun to increase exponentially, and this monograph testifies that it will continue to do so. In clinical chemistry it has never been customary to reject a method that provides useful data just because an unknown or nonspecific entity is being measured. As long as meaningful comparisons may be drawn between the values normally obtained and those from abnormal states, few questions concerning the ultimate meaning are asked, at least by the practitioner. Many of the tests of clinical chemistry fall into this category, and enzyme activities are no exception, as the contributors to this publication can attest. Providing this fact is not forgotten, no practical problem exists. However, in scanning the current literature one sees that with the increased interest in serum enzymes several assumptions are often made, usually tacitly, which in point of fact are considered to be explanations and form the basis for more and more work. These hypotheses are easily made and are sometimes supported by circumstantial evidence but, since they are not necessarily tested experimentally, they are more nearly in the nature of speculations and therefore are hazardous as bases for the explanation of observed effects or for finite projects. Three themes seem to occur most frequently: first, that enzyme activity and concentration are so simply related that concentration can be reliably inferred from measurements of activity; second, that enzymes as detected by their activity are the equivalent of simple chemically homogeneous entities; and, finally, that the kinetics that apply to one activity apply equally well to that same activity under different conditions.

In this presentation we outline the considerations in serum enzyme determinations and, consequently, we must make explicit what has been implicit in much recent work; discuss how these considerations apply in choosing between alternative methods; and describe at least one example of reducing a method to its simplest terms. When the desirable goal of both enzymatic and manipulative simplicity is thus reached, the determination of serum enzyme activity can achieve reliability even in unskilled hands.

Even in highly purified enzyme systems the catalytic activity of the enzyme is a function of *p*H, temperature, time, substrate concentration, coenzyme concentration, and the other parameters, the discussion of which makes up the four volumes of Colowick and Kaplan's standard work on the subject.[1]

FIGURE 1 illustrates the large variation of activity when a constant concentration of serum lactic dehydrogenase (LDH) is used to catalyze the diphosphopyridine nucleotide (DPN) dependent oxidation of lactic acid to pyruvic acid at different temperatures (F. Hershey, unpublished data). The reaction is measured, in the conventional manner, as the increase, as ordinates, in absorption of one of the products, reduced DPN (DPNH) versus time on the abscissa. Between 12° and 40° C. the enzyme activity has increased tenfold while the enzyme concentration has, if anything, decreased.

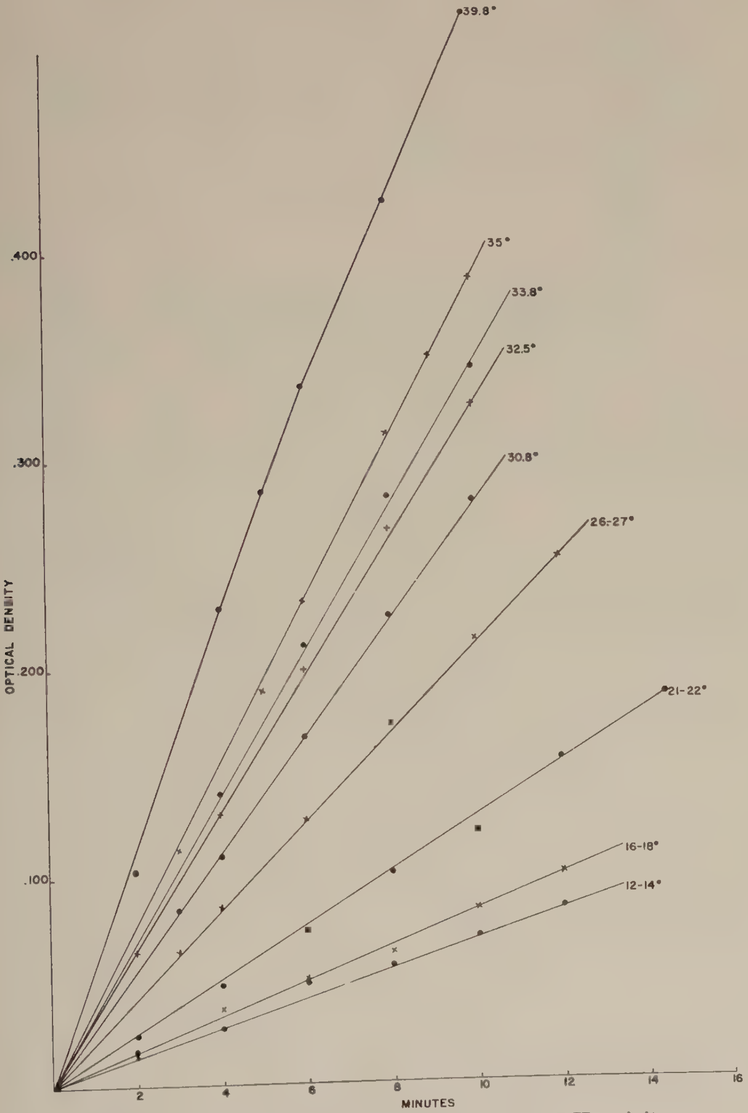

FIGURE 1. The effect of temperature on serum LDH activity.

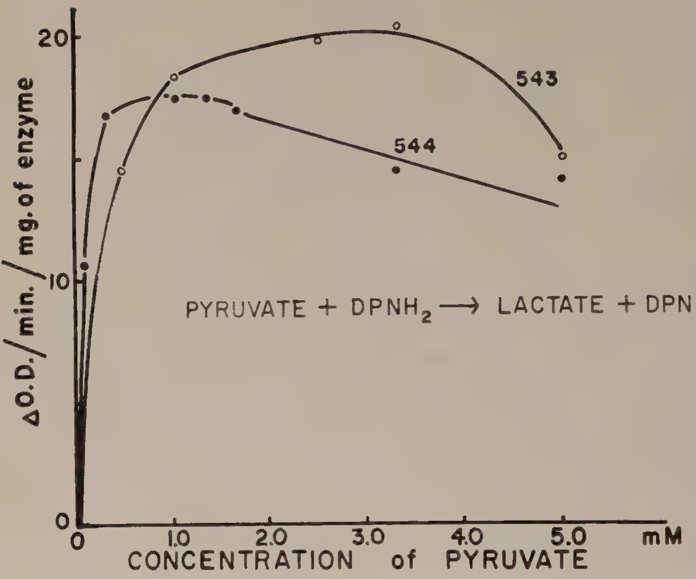

FIGURE 2. The activity of two preparations of pure LDH versus substrate concentration.

At constant temperature and other parameters, enzymes are usually considered to form a predictable system, but in fact large variations may occur even between different crystalline preparations of pure enzymes. FIGURE 2 shows the reaction rates at varying substrate concentrations for two consecutive preparations of crystalline (LDH) from the same source (W. E. C. Wacker, unpublished data). Both the activity per unit of enzyme concentration and the relationship of substrate concentration to activity are quite different. Tenfold differences have been observed in the optimal pyruvate concentration between different batches of crystalline LDH. If one were to measure only one of these curves and calculate the concentration of the other enzyme from the relative activities of the two preparations at the measured optimum, errors of 25 per cent would result, even in these pure systems.

In this system it has been proved, not only that the activity may be unrelated to the concentration, but also that the activity is not due to a single entity. Wieland and Pfleiderer[2] have shown that the reversible reduction of pyruvate to lactate in the presence of DPNH and DPN (that is, LDH activity) is a property of several proteins of any one animal organ. These proteins are quite different from each other except for their common catalytic ability in the lactate-pyruvate system. Different organs of the same species have from 1 to 6 such proteins. In turn, the analogous organs from different species contain different proteins in different numbers. Bearn and Vesel discuss these facts from yet another point of view elsewhere in this monograph. These investigators have previously shown that in human serum the LDH activity is a property of 3 apparently separate proteins that migrate with electrophoretically differentiable portions of the serum proteins. They have shown also that only certain of these activities are selectively affected after myocardial infarction and still others in myelogenous leukemia. Thus LDH activity is apparently far from being the monopoly of one protein.[3]

The kinetics of LDH may also be complex. In FIGURE 2 we saw pyruvate substrate concentration curves for crystalline LDH. FIGURE 3 shows the same information for human serum LDH, and also the DPNH substrate concentration curve. The rate of reaction is shown as the ordinate, and the pyruvate or DPNH concentration is the abscissa. A constant amount of serum was used (D. Ulmer, unpublished data). Both curves have much sharper maxima than those seen with preparations of muscle enzyme shown in FIGURE 2. This situation produces problems in the measurement of LDH in serum, because serum normally contains pyruvate ion in quite variable concentration, as shown by the cross-hatched area on the abscissa. One can see that the pyruvate is a potent inhibitor of the reaction being measured, and that the pyruvate found in serum may be at a concentration in the inhibiting range. DPNH is also an inhibitor at higher concentrations. It is such factors as these that have caused some workers to report poor results in working with serum LDH. In measuring serum LDH activity by the method usually applied to tissue or pure enzymes, one first adds DPNH, then allows 20 minutes to pass so that the pyruvate, which is endogenous to serum, will be reduced to lactate. Then the optimal amount of pyruvate is added, and the reaction is followed until a rate has been ascertained. However, an uncertain amount of DPNH is expended in the endogenous reaction so that, depending on how much is originally added, and how much has reacted, the concentration of DPNH just before the addition of pyruvate and subsequent rate measurement may be in

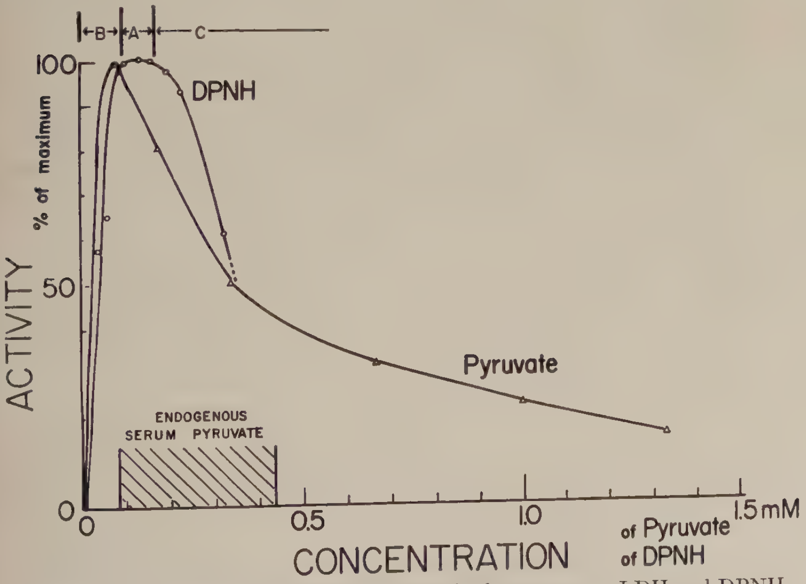

FIGURE 3. Substrate concentration curves for human serum LDH and DPNH.

any of regions A, B, and C of FIGURE 3. The reaction will be linear only when the DPNH concentration ends in region A, and then only if it is slow enough not to consume much DPNH, thereby decreasing the DPNH concentration to coincide with region B. If by chance the DPNH concentration subsequent to the "endogenous reaction" coincides with region B just at or below the maximum, the rate of the measured reaction may decrease with time as the DPNH concentration falls, as shown by FIGURE 4B (F. Hershey, unpublished data), and this will be most pronounced at higher rates. If it is above the maximum, the rate of the reaction may increase as the reaction proceeds, reducing DPNH and moving the DPNH concentration toward the maximum, as shown in FIGURE 4C. The values obtained are therefore a complex function of size of sample, concentration of endogenous pyruvate, and time of measurement.

It is important to note that many methods do not continuously monitor the reaction in question, but measure the difference in concentration of some member of the reaction only between two isolated times. These methods overlook the phenomena just described, thus introducing further uncertainty into the already tenuous relationships.

When enzymatic reactions are coupled, the relationships described above are complicated even further. Glutamic-oxalacetic transaminase (GOT) is often measured by coupling the transaminase reaction with the reduction of the product, oxalacetate, by DPNH[6] in the presence of malic dehydrogenase (MDH). The data obtained are valuable in diagnosis of many conditions, among which is myocardial infarction. Interpretation in terms of enzyme concentration subjects the results to the compounded uncertainty of two such systems as have just been described.

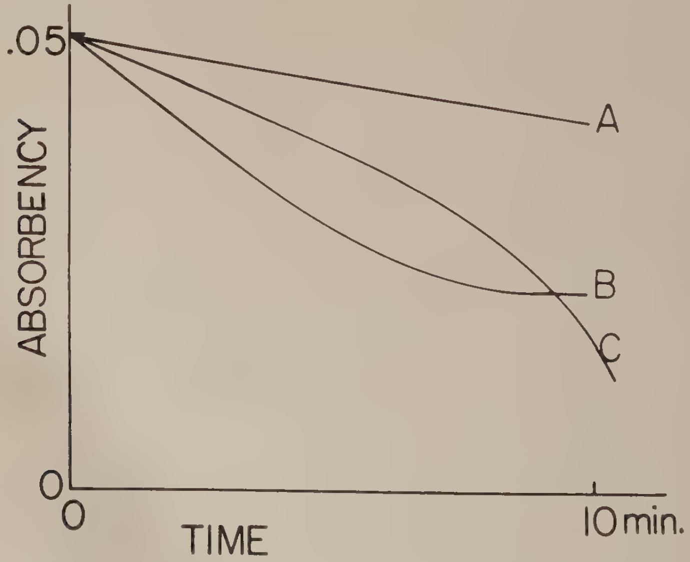

FIGURE 4. Typical rate curves for the oxidation of DPNH by pyruvate.

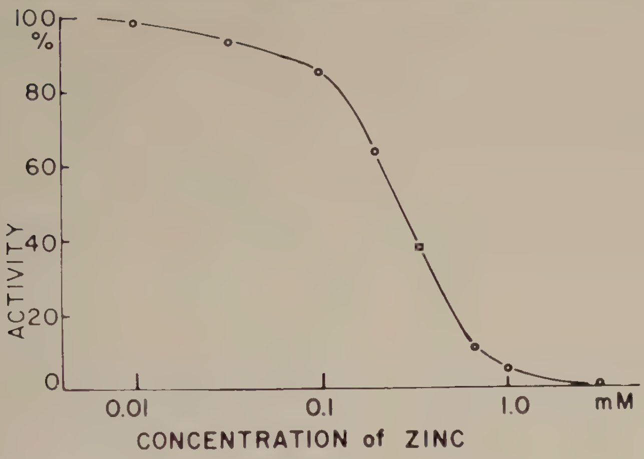

FIGURE 5. The inhibition of LDH by zinc.

The presence or absence of inhibitors such as traces of heavy metals or of activators such as manganese for isocitric dehydrogenase and pyridoxal phosphate for transaminases can also change the activity of enzymes by orders of magnitude without altering their concentration. FIGURE 5 shows the inhibition of LDH by Zn (W. E. Wacher, unpublished data). It is known that the concentration of Zn and Cu in serum changes markedly after myocardial infarction,[4, 5] and it is likely that other cations and anions also change. That such changes alter enzyme activities is well known, but the effects they have in serum are quite unknown.

It is therefore clear that in these systems none of the assumptions holds true. First, the activity of the serum enzyme is an extremely uncertain measure of enzyme concentration, itself even being inconstant; second, the enzyme activity is due to several proteins of different properties; and third, the relatively simple kinetics of the tissue enzyme shown in FIGURE 1 are in error by orders of magnitude if applied to serum.

An additional fact derives from these three, however, since they show clearly that it may well be difficult to obtain reproducible activity values from sample to sample, not to mention different concentrations. They point out the necessity for choosing a system for assaying activity that is as simple as possible and subject to as few as possible of the vagaries described if the resultant data are to be most meaningful. These considerations take on great practical importance as two, three, or more methods become available for the determination of an enzyme (or enzymes) bearing on the same diagnostic problem.

The above factors, as well as others, must be considered in deciding upon the method of choice. The effort invested in measuring these considerations is well repaid in terms of the reliability, the convenience, and the precision of the method finally chosen. Continuing to use LDH as our example, we may compare the two possible directions in which one may follow the reaction

TABLE 1
LDH ACTIVITY MEASUREMENTS

	Lactate → pyruvate	Pyruvate → lactate
Reagents:	DPN, lactate, buffer	DPNH, pyruvate, buffer
Method:	Add 0.2 ml. serum to reaction mixture	Add serum to DPNH and buffer. Wait 20 min. or until endogenous reaction ceases. Add pyruvate
Read:	At 340 mμ, 3 min.	At 340 mμ, 10 min. If rate changes with time during measurement, readjust serum concentration and repeat

illustrated in TABLE 1. In the reduction of pyruvate to lactate, a 20-min. waiting period is necessary, and the considerations described above make it difficult to obtain a linear rate, without which high precision is difficult to achieve.[6] (The GOT system, which is further complicated by the presence of a second enzyme, malic dehydrogenase, suffers from these same considerations, as well as from added complexity in manipulation.) In the oxidation of lactate in the pyruvate direction, no waiting period is necessary, and linear rates are the almost invariable rule. The reasons for this are seen in FIGURE 6, which compares the activities of a constant concentration of human serum LDH at varying concentrations of lactate and of DPN, other conditions being optimal in both cases. As in FIGURE 3, the lactate concentration normally found in serum is shown as a cross-hatched area on the abscissa. At the concentration of lactate used the endogenous lactate or other possible competing substrates have no effect on the rate, since wide variations in substrate concentration are permissible. Therefore one need not use up the endogenous substrate before measuring the activity, and consistent linear rates are observed even at very high activities. Since more DPN is used in the latter reaction than DPNH in the former, one literally pays for these advantages.

In addition to the greater simplicity of the LDH determination and the problems discussed above, comparison of the time courses of LDH and GOT following myocardial infarction make the former the method of choice. These

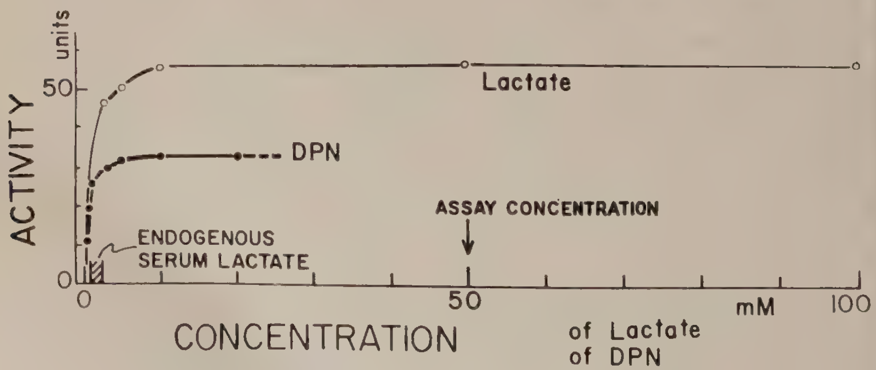

FIGURE 6. The LDH activity of serum versus substrate concentration.

time courses are shown in FIGURE 7. The upper limits of the respective normal values, 40 and 100 units, are used as a common base line, since the actual spectrophotometric precision of an individual determination is about the same for both methods. The mean values and the highest values observed in the original studies are shown.[5, 7] The activity of LDH rises to a higher value and remains elevated longer than does that of GOT. For these reasons LDH in our hands has proved to be a more sensitive and reliable, as well as a more convenient, determination for use in diagnosis of myocardial infarction. Somewhat similar considerations are said to apply to GOT and glutamic pyruvic transaminase in hepatic disorders, the latter being the method of choice.[8]

At present the practitioner faced with a possible case of myocardial infarction can determine the white cell count and sedimentation rate, look at the electrocardiogram, and consider the over-all clinical picture. When, as happens, the white cell count is equivocal, the sedimentation rate increases late in the course, the electrocardiograph has yet to show alterations, and the clinical picture is blurred, the office determination of serum enzymes would be of extreme use, provided it were carried out as simply as these other tests. The determination of pyridine nucleotides has the requisite simplicity because of the strong ultraviolet absorption of DPNH. A tiny monochromatic ultra-

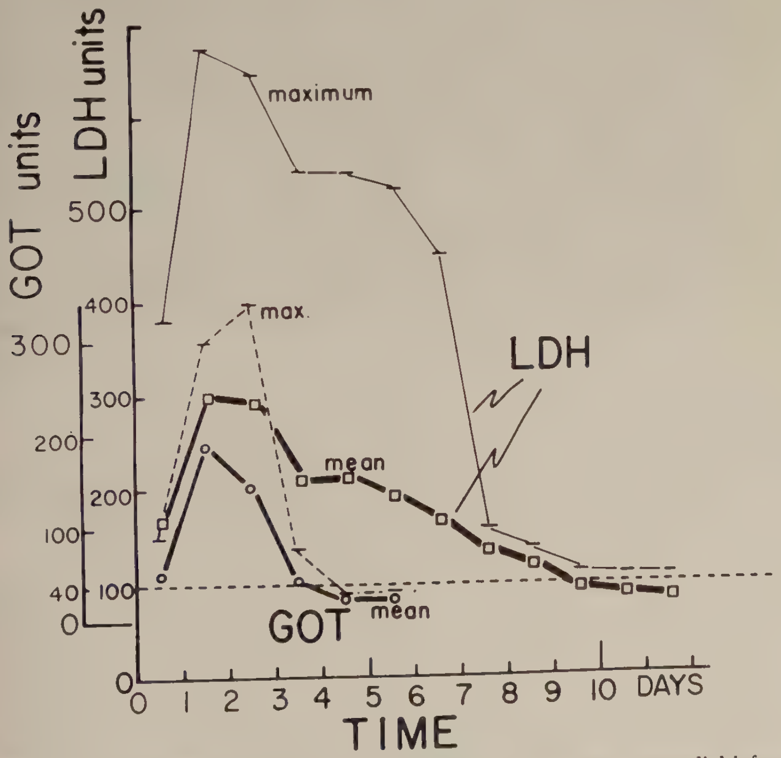

FIGURE 7. Time courses of GOT and LDH activities in serum after myocardial infarction.

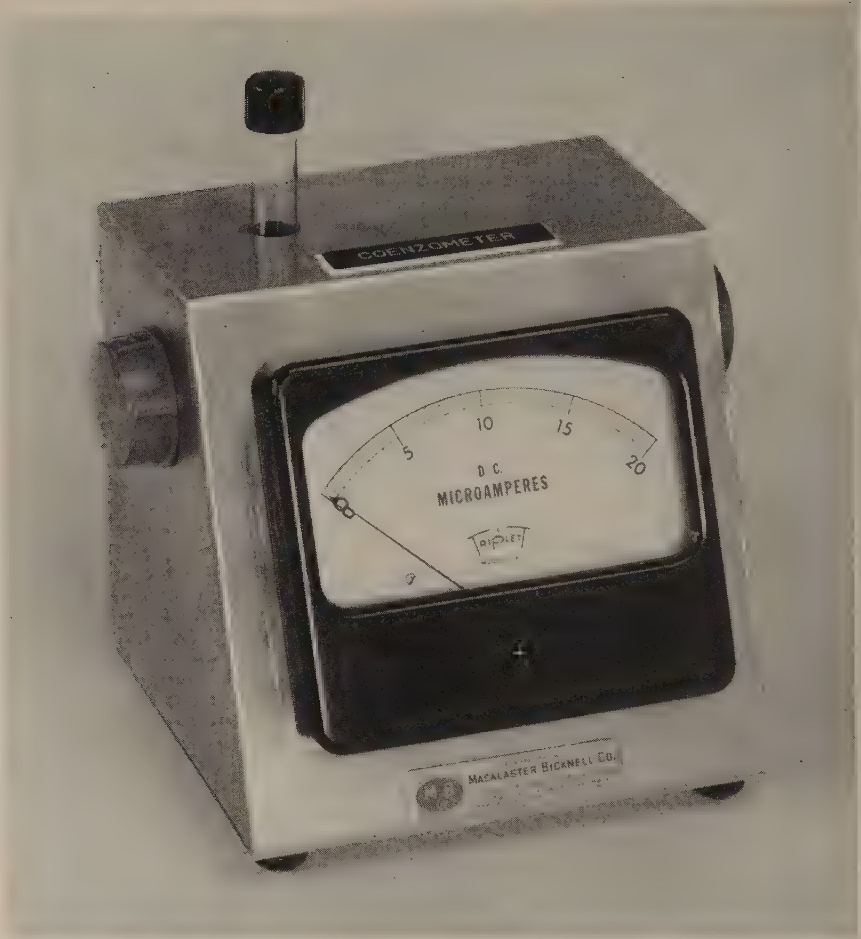

FIGURE 8. Appearance of the Coenzometer, manufactured by the Macalaster Bicknel Co., New York, N. Y.

violet colorimeter, called a Coenzometer,* has been specifically designed to give matching simplicity in the instrumentation, making the determination of serum LDH as simple as any of these tests. The use of this device, shown in FIGURE 8, will be described as facilitating the approach in question.

Lactic dehydrogenase is measured on this instrument as follows. Three-ml. aliquots of a solution containing all of the reaction components except serum are placed in each of a large number of 13-mm. diameter culture tubes that fit the instrument as cuvettes. These are then frozen and stored (for long-term storage they may be lyophilized and reconstituted; also, tubes so prepared may be purchased). For each test one tube is thawed and brought to the temperature of the instrument; then 0.2 ml. of serum is added. The mixture is used as its own blank by quickly setting zero optical density at zero time. With normal sera the meter needle moves very slowly down the scale as DPNH is formed, but with high LDH activities it moves very rapidly. By calibrating the instrument against a spectrophotometer with a 1-cm. cuvette its scale readings can be translated directly into activity units. The reproducibility of the instrument is such that the normal range for 3 min. can be marked directly on the face of the meter. Under optimal conditions its accuracy equals that of the Beckman spectrophotometer.

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* Coenzometer is the trade mark of this instrument, which is manufactured by the Macalaster Bicknell Co., New York, N. Y.

The design of the instrument is extremely simple. By fortunate chance, the light output of the ultraviolet fluorescent lamps used for illumination of fluorescing outdoor advertisements is strong, monochromatic and covers the absorption band of DPNH, as shown in FIGURE 9. The optical and electric diagram of an instrument using this lamp as source and monochromator is shown in FIGURE 10. The lamp is labeled T, and its light passes to the right through a cylindrical water-filled lens, L, through a culture tube as cuvette C, and through a variable aperture A, and falls on a photovoltaic cell P. The amount of light reaching P depends directly on the transmission of the solution S in the 350 mμ region of the spectrum. Variation of the aperture A permits setting 0 and 100 per cent transmission. Light from the lamp also passes to the left through a second variable aperture A_1 and falls on a second photocell P_1. The two photocells are connected in opposition across a 20-μamp. meter M, so that light falling on P_1 suppresses the zero of the meter scale by any chosen amount, which may be set by varying aperture A_1. By appropriate suppression of zero, the meter full-scale may be arranged to read from zero to infinity absorbency, from zero to 0.30, from zero to 0.13, or any similar fraction of the optical density range. Thus full advantage may be taken of the extreme stability of this simple circuit.

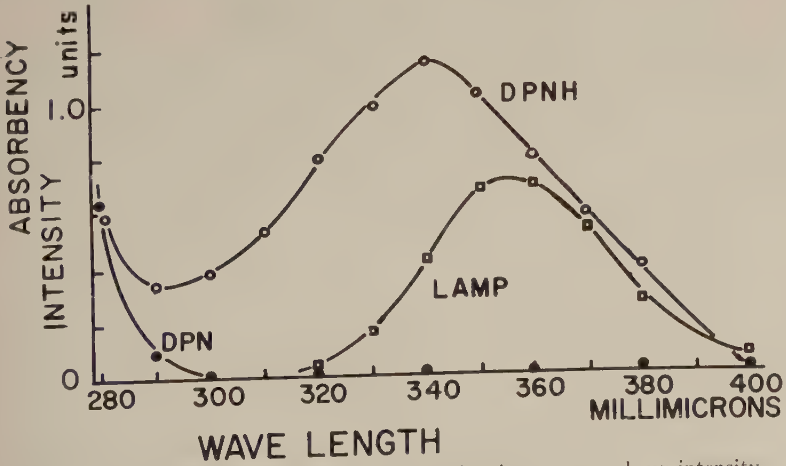

FIGURE 9. Spectral distribution of DPNH absorbency versus lamp intensity.

Calibration curves for DPNH, ammonia with Nessler's reagent, and chromium as chromate ion are shown in FIGURE 11. By similar techniques the instrument can be used for quantitative measurements of any system that absorbs at 350 mμ.

In summary, it has been emphasized that three tacit assumptions currently popular in the literature are false and may be misleading: first, enzyme activity is not a reliable measure of enzyme concentration; second, a single enzyme activity is not likely to be due to one chemically homogeneous protein; and, finally, the kinetics of one system cannot be applied safely to another system with the same activity. Methodological factors have been outlined, using as examples serum GOT and the LDH system, in which inhibition by pyruvate and DPNH make only one direction of reaction practical for reliable activity measurements. These factors are pertinent in the choice of a method for clinical purposes, where usefulness, simplicity, and reliability in the hands of technical persons unversed in enzymology are highly important. A specific

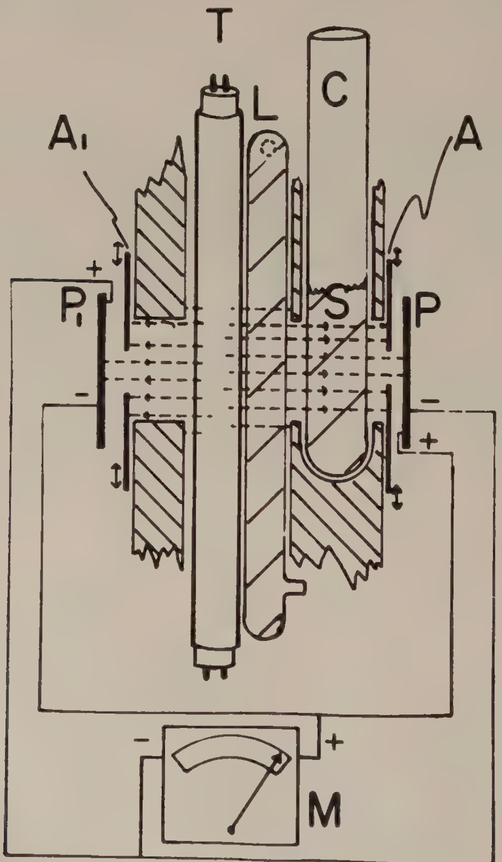

FIGURE 10. Diagram showing design of Coenzometer. See text for explanation.

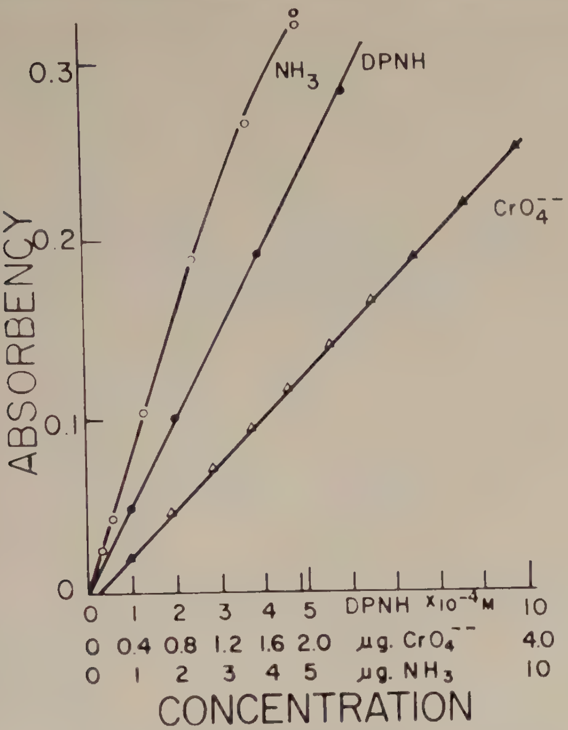

FIGURE 11. Calibration curves on the Coenzometer.

case of simplified instrumentation for the determination of serum enzymes has been described.

Acknowledgments

Our thanks are due to F. Hershey, of the Washington University Surgical Service, Veterans Administration Hospital, St. Louis, Mo., and to our colleagues W. E. C. Wacker and D. Ulmer for permission to use their unpublished data, and to the Macalaster Bicknell Co. for permission to describe the details of the Coenzometer.

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Discussion of the Paper

Burroughs R. Hill (*City of Hope Medical Center, Duarte, Calif.*): What *p*H did you use in your experiments to show the pyruvate inhibition of lactic acid dehydrogenase?

Ralph Thiers: We used *p*H 7.4.

Burroughs R. Hill: You will find that with a *p*H of 7.8 or 8.0 the inhibition is not as pronounced. In my LDH method I dilute serum 1:10 and use 0.1 ml. of this dilution. The amount of pyruvate present in this amount of serum causes no inhibitory effect and does not change the substrate concentration to any significant degree. I have not detected any inhibitory effect with DPNH.

MUSCLE MEMBRANE AS A DYNAMIC STRUCTURE AND ITS PERMEABILITY TO ALDOLASE*

By Kenneth L. Zierler

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Although the investigation I am about to report was directed at certain properties of muscle membranes, it may also be relevant to the question of why certain enzymes are present in plasma, since most enzymes appear to have no enzymic function therein. Possibly they are in transit either from a site of synthesis to a site of usefulness or from a site of usefulness to a site of disposal. With respect to glycolytic enzymes, at least, there is no evidence of synthesis in a remote tissue for use by the rest of the body. Rather, these enzymes are probably synthesized by the cell within which they work. It is likely, then, that circulating glycolytic enzymes are debris.

About five years ago we (Zierler *et al.*, 1953) noticed that when rat diaphragm, which has long been a favorite tissue for metabolic study, was incubated with hexose phosphates there was complete downward dissimilation of these metabolites to lactate. This was surprising, since earlier reports had denied that hexose phosphates could penetrate cells. As a matter of fact, it turned out that it was not necessary for hexose phosphates to enter the diaphragm. After a preliminary period of incubation the diaphragm could be removed from the medium and hexose phosphates added, and the medium would effect dissimilation of hexose phosphates down to and including lactic acid; that is, a glycolytic system complete from phosphoglucomutase to lactic dehydrogenase moved out of the diaphragm.

We began to examine some of the factors that might modify this rate of movement and elected to measure only aldolase, on the assumption that its movement was merely symbolic of the movement of a whole family of enzymes. This assumption may be incorrect, since Shaw and Stadie (1957) have failed to demonstrate any glycolytic enzyme except aldolase in their medium following preliminary incubation with rat diaphragm, although Beloff-Chain and her colleagues (1953, 1954) have found movement of phosphoglucomutase and phosphohexoisomerase. It may be that aldolase diffuses more readily than the other glycolytic enzymes.

Turning now to consideration of the movement of aldolase from excised muscle, let us first ask by what mechanism aldolase appears in the medium. There are only two possibilities: either aldolase diffuses out of muscle, or it is transported out of it. However, once aldolase appears in the medium it is conceivable that some of it may re-enter muscle, and re-entry may result either from diffusion or from some transport mechanism. The net amount of aldolase in the medium, which is the quantity measured in fact, is the differ-

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* The work reported from this laboratory was performed under Contract NR 113-241 between the Office of Naval Research, Department of the Navy, Washington, D. C., and The Johns Hopkins University. It was supported further by a grant-in-aid from the Muscular Dystrophy Associations of America, Inc., New York, N. Y. Reproduction in whole or in part is permitted for any purpose of the United States government.

ence between outward and inward movement for which there are four possibilities: (1) diffusion in both directions, (2) transport in both directions, (3) outward diffusion and inward transport, or (4) inward diffusion and outward transport. Since aldolase is probably synthesized within muscle and there is no apparent utility in its active transport, it is assumed tentatively that the movement of aldolase is simply by diffusion. We shall hereafter refer to this as efflux of aldolase, on the further assumption that inward diffusion of aldolase is negligible under our experimental conditions in which the concentration of aldolase in the medium is always very small compared to its concentration in muscle.

Changes in efflux of aldolase must be due either to changes in velocity (that is, to altered impedance to the flow of aldolase), to changes in path length, or to changes in the area of the membrane through which aldolase diffuses. None of the experimental data distinguish among these possibilities, but it is assumed that the first two are improbable (for example, it is likely that the major barrier to diffusion lies within the membrane, so that altered impedance to flow within the cell would have no measurable influence on the over-all movement of aldolase out of the cell, and changes in path length chiefly imply changes in membrane thickness), and that altered efflux of aldolase is the result of changes in the area of the membrane through which aldolase diffuses.

When paired segments of rat diaphragm were incubated either in oxygen or in nitrogen there was greater aldolase efflux under anaerobic conditions (Zierler, 1956a). The efflux of aldolase from diaphragm tissue was also increased by lack of glucose, by high potassium concentration in the medium, by transfer of diaphragm tissue to fresh medium—a maneuver that depresses metabolism (Zierler, 1956b)—and was reduced by incubation at a lower temperature. These observations suggested that permeability of muscle membrane was sensitive to the metabolism of muscle.

It remained possible that aldolase may simply have leaked from cut ends of diaphragm segments, although it was unlikely that this sort of leak would be sensitive to the factors that modified aldolase efflux. Nevertheless, it was desirable to eliminate this possibility by using a muscle whose fibers were not transected. Such a muscle is the peroneus longus of young rats. This is a somewhat fusiform muscle with long parallel fibers. It has two long tendons, so that it can be excised easily without transection of muscle fibers, and it is of sufficiently small diameter to permit adequate oxygenation of its core.

Aldolase diffused from excised peroneus longus under all conditions. The time course of aldolase efflux is illustrated in FIGURE 1. Aldolase efflux tended to increase with time in Krebs-Ringer phosphate solution.

FIGURE 2 shows that anoxia increased aldolase efflux and also that, in general, aldolase efflux varied inversely with muscle weight. Presumably, the effect of weight reflects the fact that effluent aldolase is proportional to the surface/volume ratio of muscle fibers and that, as weight increases, length increases more slowly than does fiber radius; that is, the surface/volume ratio varies inversely with muscle weight. For this reason aldolase efflux was plotted against muscle weight under a variety of conditions. Aldolase efflux was increased in a glucose-free medium and by anoxia (FIGURE 3) and by high

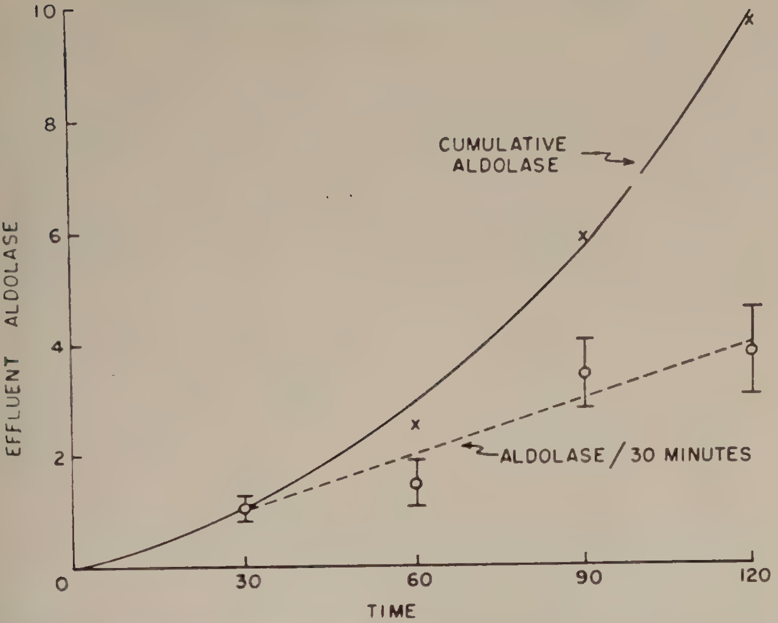

FIGURE 1. Time course of aldolase flow from rat peroneus longus. Ordinate: percentage of muscle aldolase flowing out of muscle during time period. Abscissa: incubation time in minutes. Solution: Krebs-Ringer phosphate.

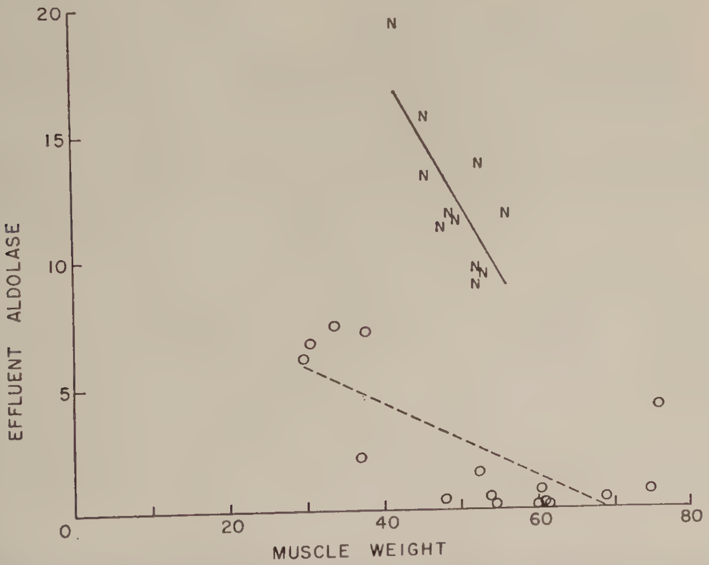

FIGURE 2. Effect of muscle weight on effluent aldolase. Ordinate: percentage of muscle aldolase moving out of peroneus longus muscle in one hour. Abscissa: mg. wet weight of muscle. Solution: Krebs-Ringer phosphate. O– – –O, in oxygen; N——N, in nitrogen. Lines are best fit to linear equation.

potassium concentration in the medium, and in all cases it was increased by transfer of tissue to fresh media. These effects are summarized in TABLE 1 (Zierler, 1957).

The effects of anoxia and lack of glucose suggest that altered metabolism of muscle produced altered permeability. This relation was examined further by observing the effects of certain inhibitors of metabolism. Iodoacetate, dinitrophenol, and cyanide all increased aldolase efflux (TABLE 2).

The effect of high potassium concentration may have been the result of membrane depolarization or may even have followed twitches induced by the

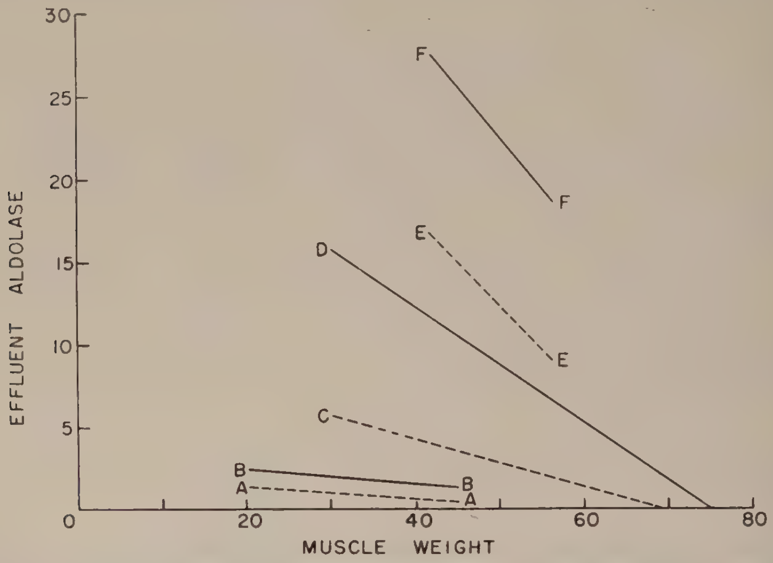

FIGURE 3. Effect of transfer of peroneus longus muscle. Muscle incubated for one hour, transferred to a second flask containing fresh solution of same composition and incubated for second hour. Dashed lines: best linear fit at end of one hour after transfer. Solutions: A and B, Krebs-Ringer-HCO_3-glucose, 95 per cent O_2, 5 per cent CO_2; C and D, Krebs-Ringer phosphate, O_2; E and F, Krebs-Ringer phosphate, N_2.

TABLE 1

ALDOLASE EFFLUX FROM RAT PERONEUS LONGUS*

No. of muscles	Medium	Effluent aldolase
		%/hr.
17	HCO_3-glucose	0.86 ± 0.10
13	PO_4-glucose	0.72 ± 0.08
16	PO_4	2.4 ± 0.7
14	High K-glucose	2.3 ± 0.3
40	High K	10.0 ± 1.1
11	PO_4 in N_2	12.5 ± 0.88
7	High K in N_2	12.5 ± 1.4

Values are means ± standard error of mean; gas phase was O_2 unless stated otherwise.
* Data from Zierler, 1957.

TABLE 2

EFFECT OF METABOLIC INHIBITORS ON ALDOLASE EFFLUX

Inhibitor	Duration of incubation	Effluent aldolase		
		Inhibitor (percentage)	Control (percentage)	Difference (percentage)
M	*min.*			
IAA 10^{-4}	90	4.9(5) ±0.8	0.38(5) ±0.06	4.5(5) ±0.8
DNP 10^{-4}	120	8.0(6) ±0.8	1.7(5) ±0.3	5.7(5) ±0.9
CN 10^{-3}	90	1.2(5) ±0.3	0.56(5) ±0.19	0.6(5) ±0.2

Data are means ± standard error of mean. Numbers in parentheses are numbers of muscles tested.

unusually high potassium concentration used. To examine the effect of recovery from stimulation, both peronei longi were exposed under pentobarbital anesthesia and one muscle was stimulated directly, supramaximally, and tetanically for 2-second intervals over a period of 10 minutes. The muscles were then excised and incubated. Aldolase efflux was increased greatly from muscles that had been stimulated (TABLE 3). Furthermore, the aldolase content of these muscles was nearly 10 per cent less than that of unstimulated paired controls, suggesting that in the intact rat aldolase diffused out of muscle to this extent during the period of stimulation or during the intervals of relaxation between stimuli.

Several qualitative statements may be made about these results. The amount of aldolase appearing in the medium is increased by factors that alter metabolism of muscle: anoxia, lack of glucose, iodoacetate, dinitrophenol, and cyanide. One possible interpretation of this effect is that there may be outward diffusion of aldolase and active inward movement of aldolase into muscle,

TABLE 3

EFFECT OF ELECTRICAL STIMULATION OF ALDOLASE EFFLUX

Medium	Duration of incubation	Effluent aldolase		
		Stimulated (percentage)	Control (percentage)	Difference (percentage)
	min.			
HCO$_3$-glucose	90 (6)	3.2 ±0.71	1.1 ±0.24	2.1 ±0.76
HCO$_3$-glucose	30 (5)	3.2 ±0.57	0.4 ±0.15	2.8 ±0.61
HCO$_3$	90 (5)	6.2 ±1.19	1.4 ±0.26	4.8 ±0.94

Values are means ± standard error of mean. Numbers in parentheses are numbers of pairs of muscles tested.

that distressed metabolism fails to support active re-entry of aldolase, and that accelerated net accumulation of aldolase in the medium results. If this is true, then the active inward movement of aldolase must be very rapid, since altered metabolism could produce tenfold changes in the rate of extracellular accumulation of aldolase. In three experiments we have examined the possibility by incubating peroneus longus in media rich in aldolase and have failed to find evidence of its rapid inward movement. Although the matter must be settled by measurement of the inward flux of labeled aldolase, it is assumed tentatively that active re-entry into muscle by aldolase is only a remote possibility, largely on the grounds that the phenomenon seems to be purposeless. It appears more likely that metabolic alterations changed membrane permeability, providing greater areas in the membrane through which aldolase diffuses.

On the basis of observed efflux of aldolase, the known diffusion coefficient of aldolase in aqueous solution, and an estimate of membrane thickness, it can be calculated that the fraction of the total muscle surface through which aldolase diffuses (if it diffuses as though it were doing so through an aqueous solution) is about 10^{-8} under conditions of least diffusion (Zierler, 1956a). It is this small fraction that is increased tenfold when one finds a tenfold increase in aldolase efflux such as occurs after anoxia.

Do these observations on excised muscle have any pertinence to events within the body? It is probable that they do. The loss of aldolase by muscles stimulated for 10 minutes *in situ* was amazingly rapid, indeed even more so than the greatest loss suffered by excised peroneus longus. On the other hand, it may be suspected that resting muscle in the body, well-perfused by normal arterial blood, may lose aldolase at a rate that is far slower than the least efflux of aldolase observed *in vitro*. This suspicion rests by analogy on observed differences between glucose uptake and insulin sensitivity of excised muscle (Zierler, 1958a) and muscles of the forearm of man studied *in situ* (Andres *et al.*, 1956; Andres and Zierler, 1956), and upon the observation of altered metabolism consequent upon mechanical handling of excised muscle (Zierler, 1956b). Indeed, the cell membrane may be one of the most sensitive regions of the cell and changes in its permeability may be the subtlest index of the production of artifacts.

It is certain that the factors reported here are only a fraction of those that alter permeability. Under appropriate conditions, for example, insulin increases permeability to aldolase (Zierler, 1958a), and permeability to aldolase is increased in mice with hereditary muscular dystrophy (Zierler, 1958b). There must be many other agents that operate upon the cell membrane to alter permeability. Such changes in permeability have the effect of varying in degree the isolation of a cell from its environment, a device that appears to be an effective regulatory mechanism for cellular metabolism. Permeability is increased during periods of hypoxia and following muscle contraction, which permits a more rapid efflux of end products and a more rapid influx of needed substrates. The efflux of aldolase may be regarded as a demonstration of incomplete specificity of membrane permeability and, because muscles in the body are exposed frequently to hypoxia and stimulation, the turnover of muscle aldolase may prove to be rapid. In any event, glycolytic enzymes circulating in blood plasma probably represent to a large degree efflux from muscle resulting at least from transient hypoxia and recovery from contraction.

Finally, the lability of the property of permeability of muscle membrane argues against the existence of membrane pores as fixed anatomical structures. How, then, does aldolase diffuse out of muscle?

It has been calculated (Zierler, 1956a) that the average area per diaphragm fiber through which the least diffusion of aldolase occurred was about 0.03 μ^2. Essentially the same area is available on each fiber of peroneus longus for diffusion of aldolase. If the area through which a molecule of aldolase diffuses must be no less than 3 aldolase diameters wide, then there can be no more than about 30 diffusion areas per fiber, either of diaphragm or of peroneus longus. In a 20-mm. fiber the mean distance between centers of such diffusion sites would be more than 0.5 mm.

Although this large distance is of no importance so long as only glycolytic enzymes are involved, an interesting bit of speculation arises when one compares rates of efflux of aldolase and potassium. The diffusion coefficient of potassium is about 20 times that of aldolase in aqueous media. If potassium diffused through many more membrane sites than did aldolase, the rate constant for potassium efflux should be more than 20 times that for aldolase efflux; however, if different experiments can be compared, it is not. For rat diaphragm, the rate constant for potassium efflux reported by Creese (1954) is only about 10 times that estimated for aldolase efflux from rat diaphragm. For frog sartorius, to use for comparison a muscle with intact fibers, the rate constant for potassium efflux reported by Keynes (1954) is only about 20 times that estimated for aldolase efflux from peroneus longus. Until measurements of potassium and aldolase efflux have been made simultaneously from the same preparation, these data imply that potassium and aldolase diffuse through the same small number of widely-spaced sites. If these sites were fixed at a mean interval of 0.5 mm. there should be saltatory conduction along muscle, which is presently believed not to occur. The problem could be resolved if the muscle membrane were in rapid motion, during which sites sufficiently large for diffusion opened up now here and now there. If these transient sites were scattered sufficiently rapidly over the membrane, even though at any instant only about 30 sites were available, the effective distance between sites might be very much less than 0.5 mm. Conceivably, this motion of the membrane, which must be more than thermal agitation, may be linked to cellular metabolism, and it may be that altered metabolism produces altered permeability by slowing the membrane dance.

Acknowledgment

I gratefully acknowledge the assistance of Ellen Rogus in measuring aldolase efflux.

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SERUM ENZYMES IN THE PHYSIOPATHOLOGY OF MUSCLE

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In 1949 Sibley and Lehninger, in a general study on serum aldolase, reported an increased activity in two patients with progressive muscular dystrophy (PMD). We thought then that an elevation in serum enzymes might represent an interesting basis for the diagnosis and, perhaps, for the search for a biochemical lesion of this disease, and we undertook a systematic survey. We studied in turn: aldolase (1953), phosphohexoisomerase (1954), transaminases (1955), and lacticodehydrase (1957). We could show that such enzyme determinations undoubtedly justify a clinical interest and allow a description of certain factors involved in prognosis. They also give rise to hypotheses on the physiopathology of PMD.

TECHNIQUES

Aldolase is determined by measuring the colored reaction of triose phosphates with dinitrophenylhydrazine (Sibley and Lehninger). Its activity is expressed in Meyerhof units (milligrams of triose phosphates produced per minute at 38° C., referred to 1 l. of serum or plasma). One unit equals 21.6 of Sibley and Lehninger's units.

Phosphohexoisomerase is determined by measuring the colored reaction of fructose-6-phosphate with resorcine hydrochloride (O. Bodansky). Results are expressed in milligrams of fructose formed in 30 min. by 1 l.

Lacticodehydrogenase is determined spectrophotometrically (Wróblewski and La Due). Results are expressed in the spectrophotometric units of these authors.

Transaminases were first determined chromatographically (Karmen *et al.*, 1955), then by spectrophotometry (Karmen), and by colorimetry (Tonhazy *et al.*; Reitman and Frankel). Results are expressed in millimoles of glutamic acid formed per liter and per hour at 38° C. One unit equals 33 spectrophotometric units.

RESULTS

Results are shown in FIGURES 1 to 10 and TABLES 1 to 5.

Value for the Diagnosis

All enzymes that we have determined display a higher activity in PMD patients than in healthy subjects (FIGURES 1 to 4). The percentage of abnormal values reaches 80 to 90 per cent for aldolase, lacticodehydrase, transaminases (both glutamic oxalacetic and glutamic pyruvic). The mean elevation is 500 to 1000 per cent. Phosphohexoisomerase results are somewhat less consistent. There is a relationship between different enzymes in the same patient. In 30 cases we found a positive correlation (r = 0.580) between aldolase and glutamic pyruvic transaminase. The increase in serum enzymes activity has been established. Since our first description of it, this increase has been confirmed by many authors (Jacob and Neuhaus; Mason and Cook; Beckmann; Aronson and Volk, 1956; Siekert and Fleisher; Pearson; Ritter and Seligson; White and Hess; and Evans and Baker). It represents, insofar as we are aware, the first biochemical symptom of PMD to be described in the blood. It seems even to be, within the limits of muscular diseases, the first biochemical test to be nearly specific for PMD. While creatinuria is common

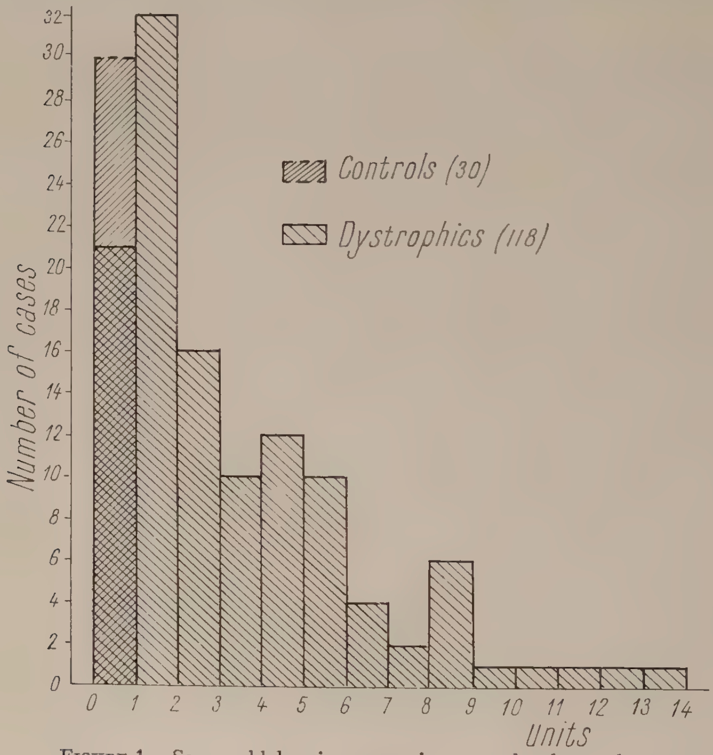

FIGURE 1. Serum aldolase in progressive muscular dystrophy.

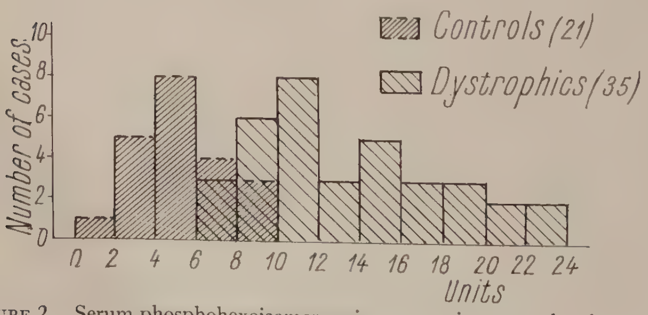

FIGURE 2. Serum phosphohexoisomerase in progressive muscular dystrophy.

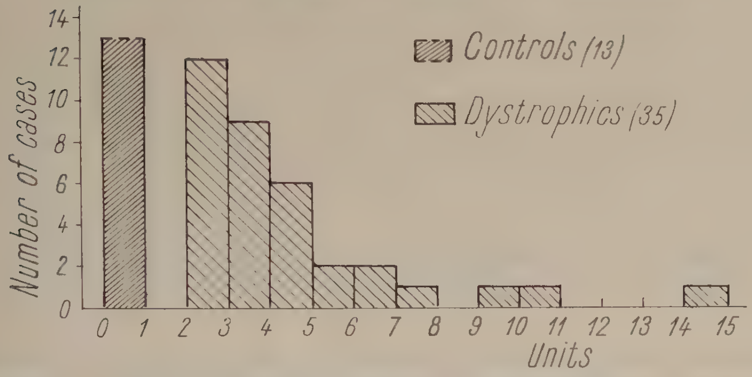

Figure 3. Serum glutamic pyruvic transaminase in progressive muscular dystrophy.

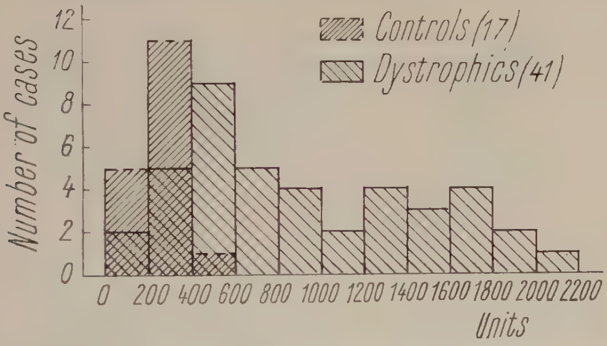

Figure 4. Serum lacticodehydrogenase in progressive muscular dystrophy.

to all muscular diseases (Milhorat), we could show that hyperaldolasemia is either lacking or very slight in diseases of nervous origin, such as poliomyelitis, amyotonia congenita, progressive spinal atrophy, and also myotonia atrophica (FIGURE 5, TABLE 3). Confirmatory results have been obtained by others (Siekert and Fleisher; Pearson).

However, such an elevation can take place in some other diseases with muscular involvement. It is observed in myositis and is particularly striking in dermatomyositis (FIGURE 5; see also Siekert and Fleisher). In hypothyroidism minor elevations have been described (Sibley and Fleisher). With R. Joseph we found a very high aldolasemia (6 units) in one infant having myxoedema and a big muscular hypertrophy. Both clinical and chemical abnormalities disappeared after therapy.

Two circumstances are of special interest for diagnosis:

When the disease is progressing very slowly, the diagnosis of PMD is sometimes questioned. In some cases in which clinicians and biochemists had given conflicting diagnoses it was finally established that the diagnosis of the biochemists was correct.

Enzyme determinations sometimes allow a very early diagnosis when the

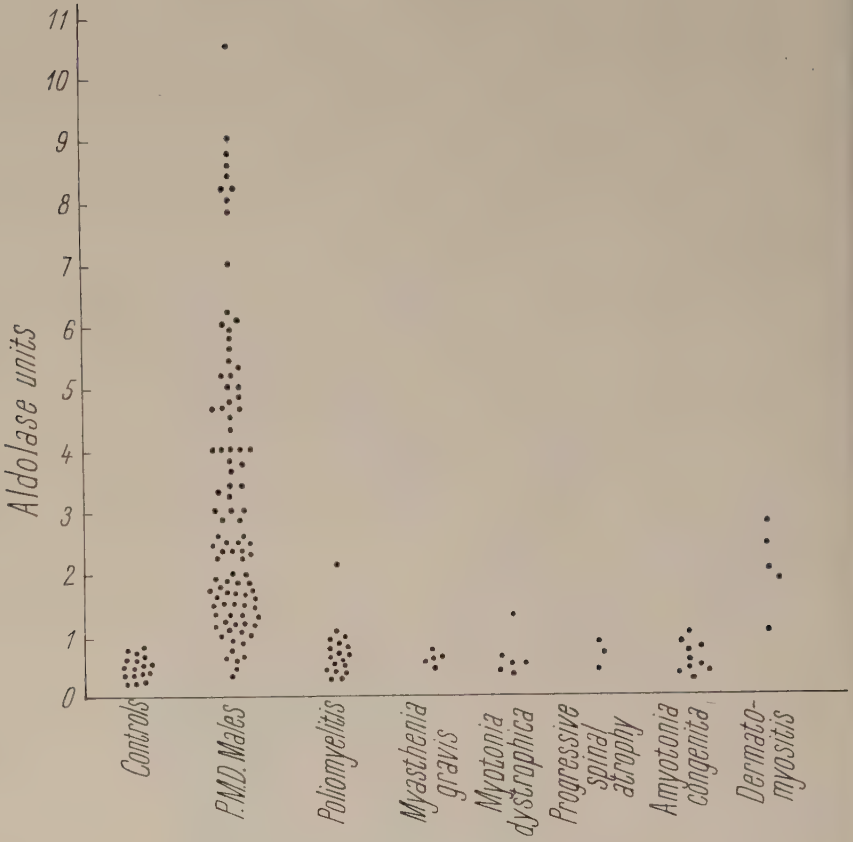

FIGURE 5. Serum aldolase in various diseases.

clinical state is still doubtful, particularly in brothers of patients with PMD. This possibility also has been reported by others (Pearson).

*Value for the Prognosis**

The level of serum enzymes is subject to variations according to many factors, as shown by the analysis of determinations of aldolasemia in 130 patients, among whom many have been seen several times.

Age. Aldolasemia is higher in young patients (under 15 years) than in adults (FIGURE 6). The difference is striking, but normal values are also significantly higher in children. Therefore, this observation is difficult to interpret.

Sex. Female patients are rare (18 of 130) and in 13 cases show normal or only slightly elevated values. Although the clinical course is often slower in girls this is not sufficient to explain the observed differences (FIGURE 7).

* F. Schapira *et al.*, 1957a.

Clinical form. Aldolasemia is higher in the low, pseudohypertrophic form than in the facial type.

Evolution. This is a prominent factor (FIGURE 8). Enzyme activity in serum is not proportional to the degree of the illness but, on the contrary, is highest at the beginning of the evolution, while the clinical state is still satisfactory. It is low in bedridden patients. In one patient the decrease of aldolasemia with years is a constant evolutional feature. A rapid evolution can be feared when one finds a high activity in a child still clinically in good condition. This holds true for children, but is less evident in adults. Adults may show very slow evolutions with little disability, and their aldolase level is often low.

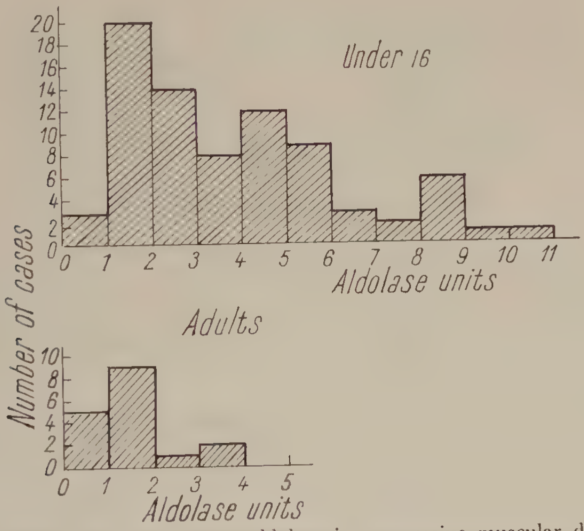

FIGURE 6. Effect of age on serum aldolase in progressive muscular dystrophy (male patients).

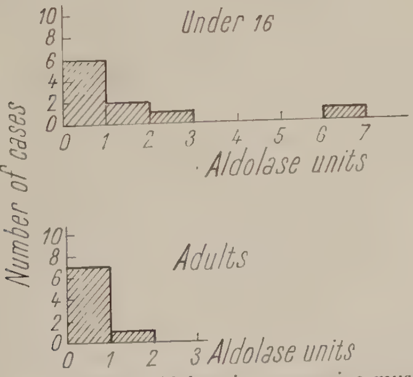

FIGURE 7. Effect of age on serum aldolase in progressive muscular dystrophy (female patients).

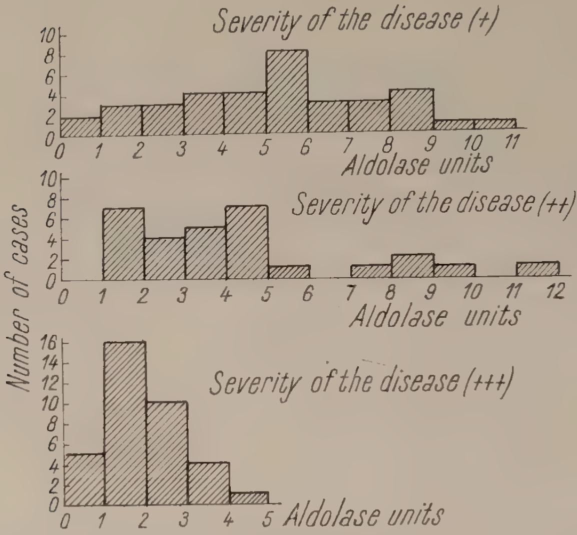

FIGURE 8. Effect of severity of the disease on serum aldolase in progressive muscular dystrophy.

Time of the year. (FIGURE 9). On the whole, we find higher values during the early, cold months (October to December) than in the warm ones (May to July). The difference is large in ambulatory subjects (severity of disease +). It disappears in bedridden patients (severity of disease +++). This influence of the temperature suggests the role of the circulation in PMD, which has been confirmed in part by arteriographic studies (Demos and Ecoiffier).

Similar studies have been conducted independently by others with analogous results (Aronson and Volk, 1957). Observations using glutamic oxaloacetic transaminase (Pearson) are partly at variance with ours regarding the relationship between the degree of illness and the level of serum enzyme. This discrepancy is probably due to the fact that their material encompasses more adults than ours, a factor whose importance has been stressed previously (Rowland).

Enzymes here described do not exhaust the list of the possible abnormalities in PMD. Preliminary results seem to indicate that malicodehydrogenase may also be elevated (unpublished data).

The increase of serum enzymes in PMD is by no means general. We could show* that all glycolytic enzymes (from the stage of fructose diphosphate) can be found in the normal serum, using fructose diphosphate as a substrate and lactic acid as the end product of the reaction. Up to now, no significant differences could be demonstrated in PMD, but it may be that our technique does not permit true quantitative estimations. On the other hand, negative results were obtained with alkaline and acid phosphatases (Luzzatto and Casirola; White and Hess), dipeptidases (Smith *et al.*), and desoxyribonuclease (our own unpublished data).

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* Unpublished results (TABLE 4).

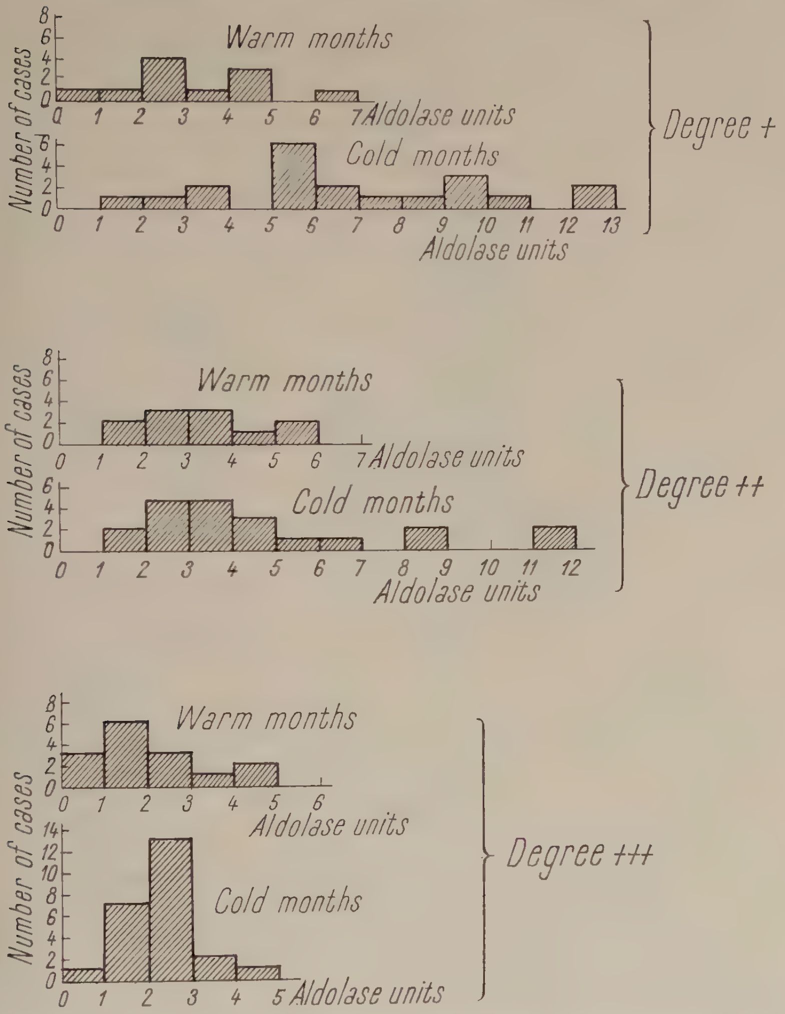

FIGURE 9. Seasonal variations of serum aldolase in progressive muscular dystrophy.

Serum Enzymes and Plasma Fractions

Zone electrophoresis allows the separation of serum proteins into discrete fractions; it is then possible to discover how the serum enzymes are transported and if new and perhaps characteristic patterns appear in any disease. This has actually been made for lacticodehydrase in the normal and in myocardiac infarction (Vesell and Bearn). We are attempting to do it with J. Rosa for aldolase and transaminases in normal and dystrophic subjects after electrophoresis on starch blocks.

Our results are still preliminary. They show that, normally, glutamic oxalacetic transaminase is located almost exclusively on the alpha-2-globulins,

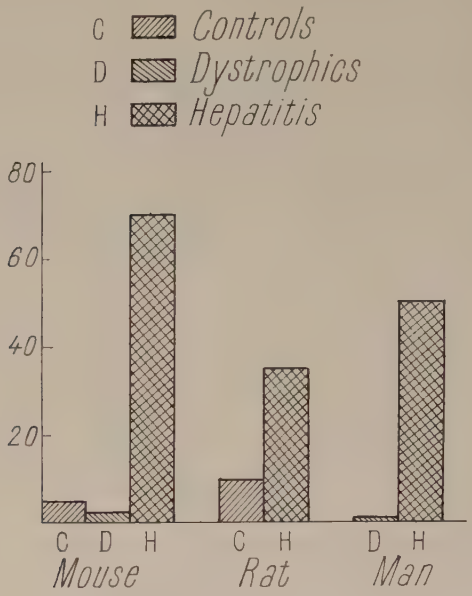

FIGURE 10. Ratio $\frac{\text{F-1-P aldolase}}{\text{FDP aldolase}} \times 100$ in various species and conditions.

and this localization does not change in dystrophy. Aldolase, on the other hand, is distributed between beta- and gamma-globulins.

DISCUSSION

Many problems arise from the observation of an increase of serum enzymes in a disease. The first one is the tissue origin of the enzymes. It is all too easy to assume in any disease that the enzymes arise from the diseased tissue itself. That this is so in PMD seemed nearly certain to Meyerhof, quoting the two cases of Sibley and Lehninger (Meyerhof) in support of this view. In actual fact, such an assumption has seldom been proved. In order to gather evidence, we have been working along several lines.

TABLE 1
SERUM ENZYMES IN PROGRESSIVE MUSCULAR DYSTROPHY

Serum enzymes	Controls		PMD	
	Number of cases	Mean	Number of cases	Mean
Aldolase (male children)	16	0.4 ± 0.035	79	3.7 ± 0.265
Phosphohexoisomerase	21	5.5 ± 0.5	34	12.6 ± 0.85
T. G. O. transaminase	8	1.3 ± 0.13	30	4.3 ± 0.34
T. G. P. transaminase	13	0.72 ± 0.06	36	3.8 ± 0.4
Lacticodehydrogenase	14	276 ± 24	22	1045 ± 250

TABLE 2

FACTORS OF HYPERALDOLASEMIA IN PROGRESSIVE MUSCULAR DYSTROPHY

Factors	Aldolasemia		Significance
Age (male patients)	<16 years	adults	
	3.7 ± 0.265	1.46 ± 0.23	<0.01
	♂	♀	
Sex <16 years	3.7 ± 0.265	1.25 ± 0.36	<0.01
Sex adults	1.46 ± 0.23	0.70 ± 0.19	<0.001
Severity of the disease (male patients <16 years)	Degree (+)	Degree (+++)	
	5.07 ± 0.42	1.93 ± 0.16	<0.001
	Cold months	Warm months	
Seasons Degree (+)	6.7 ± 0.7	3.0 ± 0.5	<0.001
Seasons Degree (++)	4.7 ± 0.68	3.3 ± 0.43	>0.05
Seasons Degree (+++)	2.2 ± 0.19	1.9 ± 0.26	>0.05

TABLE 3

SERUM ALDOLASE IN VARIOUS MUSCULAR DISEASES

Disease	Number of cases	Aldolasemia
Progressive muscular dystrophy (male patients)	79	3.7 ± 0.265
Myotonia dystrophica	6	0.48 ± 0.1
Amyotonia congenita	10	0.60 ± 0.2
Myasthenia gravis	5	0.47 ± 0.07
Polyomyelitis	9	0.48 ± 0.06
Progressive spinal atrophy	3	0.40
Dermatomyositis	5	2.0 ± 0.13

Release of Enzymes from Muscles in PMD

Determination of enzymatic activities in muscle biopsies. A considerable decrease in the activity of several enzymes of glycogenolysis is shown by determining the enzymatic activities in muscle biopsies (Dreyfus *et al.*, 1956). The over-all reaction of glycogenolysis itself is lowered. This decrease holds true when referred to muscle protein itself and not to the net weight of the tissue (Dreyfus *et al.*, 1954). Such a decrease may explain why aldolasemia is declining with time: we may admit that a given part of the total muscle mass enters the blood stream in one unit of time. At the end of the evolution, the enzyme content of the tissue has become very low.

Determinations of aldolase attempted in arterial and venous femoral plasma. These determinations were not made in healthy subjects,* since the normal activity is too low to expect significant results. They were performed in rabbits by inserting a catheter into a femoral vein and artery. No significant differences were found. However, samples of arterial and venous blood were

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* After muscular work in healthy subjects no change in aldolasemia occurs.

TABLE 4

SERUM GLYCOLYSIS (MILLIGRAMS OF LACTIC ACID FORMED IN 1 HOUR PER 100 ML.)

	Controls	Dystrophics
	3.2	6.5
	3.3	1.4
	−2.6	10.0
	2.7	1.0
	9.9	0.0
	4.0	5.6
	4.0	14.0
	6.8	7.5
	2.5	3.0
	3.4	8.5
	6.4	1.0
	−0.8	
	3.0	
Mean:	3.6 ± 0.86	5.3 ± 1.3

$P > 0.05$

TABLE 5

ALDOLASE OF ARTERIAL AND VENOUS PLASMA IN PROGRESSIVE MUSCULAR DYSTROPHY (UNITS)

Arterial plasma	Venous plasma	Difference V–A
1.05	1.1	0.05
2.1	2.1	0.0
1.1	1.1	0.0
1.55	1.55	0.0
1.3	1.3	0.0
0.1	0.1	0.0
1.4	1.6	0.2
3.65	4.15	0.5
1.75	1.9	0.15
1.4	1.6	0.2
		Mean: 0.11

obtained in 10 dystrophic patients. In 5 cases, the values were identical; in the other 5, values were slightly higher in the vein than in the artery (TABLE 5). The difference is statistically significant, but the application of statistical tests is not quite correct, since the distribution is not gaussian. At any rate, it appears that, in some cases at least, delivery of aldolase from muscle to plasma has been directly proved.

1-Phosphofructoaldolase. It has been shown repeatedly (Hers and Jacques, 1953; Leuthardt *et al.*, 1953; Ta-Chen-Tung *et al.*, 1954) that there exist two types of aldolase in tissues. The activity of one type is limited to fructose diphosphate (FDP). The other type is active also on compounds that can be split into phosphodihydroxyacetone on the one hand, and a series of nonphosphorylated aldehydes on the other. The best known of these compounds is fructose-1-phosphate (F-1-P). It has been found, when working with substrates at saturation, that muscle aldolase is about 100 times more active on

FDP than on F-1-P, while the liver enzyme is nearly as active in both substrates (Hers, 1957). Consequently, we can hope to trace the origin of serum aldolase by inquiring into its action upon F-1-P. We worked first with a sample given us by H. G. Hers; subsequently we synthesized F-1-P. We found (Schapira *et al.*, 1957b) that in human PMD and in dystrophic mice the serum is active only on FDP. In patients with hepatitis, as well as in rats and mice poisoned with carbon tetrachloride, the activity is only slightly less on F-1-P than on FDP (FIGURE 10).

Studies on that subject are presently in progress. A survey of rat tissues shows that F-1-P aldolase is present in liver, kidney, and intestine, and nearly absent from muscle, heart, brain, and red cells. This last point may be helpful in eliminating the role of hemolysis, which may sometimes obscure the results, since the activity of most enzymes is many times higher in cells than in plasma.

This type of research may give an idea of the tissue origin of enzymes in normal serum. An attempt to establish this was unsuccessful in man because the aldolase activity is too low, and no activity against F-1-P could be detected. It could be undertaken in rats and mice, whose serum activity is respectively 10 and 20 times higher than that of men. In such conditions the ratio

$$\frac{\text{aldolase FDP}}{\text{aldolase F-1-P}}$$

was about 10 in rats and in mice.

This indicates the presence of both types in normal serum. Assuming from the results in hepatitis that the "liver aldolase" is approximately half as active on F-1-P as on FDP, we can deduce that the "liver enzyme" contributes about 10 to 20 per cent of the serum aldolase in the rat and in the mouse in the normal condition.

The mechanism of release. Comparison of PMD with other syndromes raises many conflicting points.

Hyperaldolasemia is found in PMD, in experimental dystrophy due to avitaminosis E (Beckmann and Buddecke) and in the hereditary dystrophy in mice (Schapira *et al.*, 1957c). However, while in muscle from dystrophic patients (Dreyfus and Schapira, 1954) or mice (Weinstock *et al.*) enzyme level is decreased, it is increased in nutritional muscle dystrophy (Beckmann and Buddecke). From a biochemical point of view, the hereditary dystrophy of mice and the clinical disease display many similarities, while nutritional dystrophy behaves in a very different way (Schapira and Dreyfus, 1955).

On the other hand, tissue aldolase is decreased in both muscular dystrophy and muscular atrophy from nervous origin. Very often, the rate of muscle wasting is much faster in nervous atrophy, as in poliomyelitis at the atrophic stage. However, in such cases there is practically no increase in serum enzymes (Schapira *et al.*, 1953; Pearson). We have attempted, unsuccessfully, to detect an increase in aldolase in venous blood taken from a denervated muscle in the rabbit as compared with arterial blood (unpublished data). Still, a moderate increase in aldolasemia was observed after nerve section in the dog (Aronson and Volk, 1957). In addition, a transitory rise occurs after minor operations and attacks of myoglobinuria (Pearson *et al.*).

An increased destruction of muscular tissue is therefore not sufficient by itself to explain an increase in serum enzyme activity. What, then, can be the mechanism of the release of proteins, which are apparently in the native state, into the blood stream?

An increase of permeability may exist in the muscles of children suffering from PMD. It is known (Zierler) that if a rat diaphragm is incubated in Ringer's solution, aldolase and other enzymes diffuse into the surrounding medium. We could confirm this for muscle, but red cells shaken in Ringer's solution or plasma do not lose any aldolase (unpublished results). The possibility of a vascular factor is supported by the increase of aldolasemia in winter and the decrease in rate of the circulation shown by arteriography (Demos and Ecoiffier). The possible role of anoxemia in favoring the escape of tissue enzymes has been stressed (Hess and Raftopoulo). Indeed, the oxygen consumption of dystrophic muscle *in vivo* is very probably diminished: with J. Demos we found the arteriovenous difference in oxygen of femoral blood to be 3.34 ± 0.4 per cent in 10 dystrophic patients as against 6.6 ± 0.6 per cent in 10 controls ($p < 0.01$—unpublished data).

Another hypothesis may be raised. We described experiments that suggest the existence of a life span of the myofibrils (Dreyfus *et al.*, 1957). If there were also a life span of the whole cell, or of the sarcoplasm, its shortening could result in an increased liberation of soluble enzymes.

We have still to consider the question involving a hormonal control of aldolase liberation. An experimental approach to this problem has been attempted by way of adrenocortical hormones. ACTH and cortisone are able to provoke an increase in serum aldolase by 200 to 1000 per cent in the rat and more easily in the rabbit (F. Schapira). This effect could not be duplicated in healthy men with ACTH, probably because the injected doses were too low. In the literature, we could find only scattered data on the effects of cortisone and of aspirin on aldolase and transaminase (Nydick *et al.*; Manso *et al.*). Such data always refer to patients, and the results are very variable. Indeed, in dermatomyositis, cortisone constantly lowers the elevated aldolasemia (unpublished results from our laboratory; also Pearson). But the effects are very likely to be different on patients and on healthy people. It may be noted that a myopathy has been induced by giving cortisone to rabbits (Ellis). The hyperaldolasemia produced by cortisone prompts us to think that an increase of enzymatic activities in the plasma could be caused by another factor than the destruction of the diseased tissue itself. Two possibilities can be suggested: enzymes could come from other tissues under the stress of the disease; the hypothesis has been made that serum aldolase in tumor-bearing rats could come from muscle (Warburg and Christian). More generally, hyperaldolasemia elicited by cortisone indicates that the enzyme could pass into the blood as a result of a general reaction of the organism to stress. This could account for hyperaldolasemia after a sham operation with little muscular damage or even play a role in hypertransaminasemia of myocardial infarction. It could explain, totally or in part, the increase in aldolase activity shown to be constant in delirium tremens (Sibley and Fleisher; Dastugue *et al.*) or the fact that transaminase increases much less after crush injury than after tourniquet shock (Albaum and Milch).

SUMMARY

Increase of plasma enzymes in progressive muscular dystrophy. This factor was studied in more than 100 patients. We described the clinical value of hyperaldolasemia (1953), and increases in phosphohexoisomerase (1954), transaminase (1955), and lacticodehydrase (1957). This was confirmed by others. The value for diagnosis is certain, since the increase is nearly constant, while lacking or being weak in muscular diseases of nervous origin. However, it exists in dermatomyositis. Hyperaldolasemia is found in dystrophic mice.

Factors of this elevation. Age, sex, evolution, and season all have a bearing on hyperaldolasemia, which is higher in children, is often lacking in girls, decreases with time, and is higher in winter.

The over-all picture evokes the role of a vascular factor.

Muscular origin of the increase. This origin seems probable, but has not been proved. We worked along three lines:

(1) Determination of muscular enzymes in biopsies shows the low level of glycogenolytic activity in dystrophy, even when taking account of the increase of fat and collagen.

(2) Aldolasemia in arterial and venous plasma was determined. In 5 of 10 cases the activity was higher in the vein.

(3) 1-Phosphofructoaldolase. Extracts of liver and kidney, but not of muscle, display an aldolase-type activity against fructose-1-phosphate. We could not detect it in the sera of normal men or in that of dystrophic patients or of mice; it is present in large amounts in the sera of mice and rats poisoned with carbon tetrachloride and of patients with hepatitis. Studies of substrate specificity may allow an investigation of the origin of a plasma enzyme.

The mechanism of the increase. This factor could be a trouble of muscular permeability or of the rate of synthesis or degradation of muscle proteins. It may be under hormonal control, as the hyperaldolasemia elicited by cortisone suggests.

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REACTIVE CHANGES OF ENZYME ACTIVITIES IN SERUM AND LIVER AS SYMPTOMS OF "ACUTE SYNDROME"

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In 1954 LaDue *et al.* made the interesting and important observation that a rise in glutamic-oxalacetic transaminase activity takes place in the serum of patients following myocardial infarction. Since that time numerous authors (Chinski *et al.*, Hsieh and Blumenthal, Kattus *et al.*, LaDue and Wróblewski, Ostrow *et al.*, Siegel and Bing) have dealt with the behavior of serum enzymes after myocardial infarction.

We investigated the respective activities of glutamic-oxalacetic transaminase (GOT), lactic dehydrogenase (LDH), aldolase (ALD), tributyrinase (TRI), and cholinesterase (CHE) in serum (Hauss, Leppelmann and Süllmann, Hauss and Leppelmann). We were concerned primarily with the problem whether the change in serum enzyme activities represents a specific symptom of myocardial infarction or whether this change is merely a nonspecific symptom of general occurrence in acute diseases attending the "acute syndrome", as it was termed by Hauss, including high temperature, leukocyte increase, changes in hemogram, increase in blood sedimentation rate, blood sugar and rest nitrogen, and variations in serum protein.

FIGURES 1 to 4 demonstrate our findings in cases of myocardial infarction, operative intervention, and infectious diseases. In these figures the normal range (mean ± standard deviation) has been illustrated by hatching.

The characteristic behavior of GOT, LDH, ALD, TRI, and CHE in the serum of a patient with myocardial infarction is shown in FIGURE 1. GOT, LDH, and ALD rise after such an attack, while TRI and CHE fall. All enzyme concentrations return to the normal range within 2 to 3 weeks.

The means of the postoperative serum enzyme activities in 18 patients are shown in FIGURE 2. It will be seen that the activities of LDH and ALD rise after operative intervention, while those of TRI and CHE fall. A statistical evaluation (*t* distribution of Student) served to demonstrate that the observed differences are not the result of chance. The change in serum enzyme levels was both independent of the type of operation (inguinal herniorrhaphy, trepanation, nephrectomy, laparotomy, or lobectomy) and also independent of the type of narcosis (ether, nitrous oxide, potentiated anesthesia, or local anesthesia). We did not determine the GOT activities after operation. However, it is known that the GOT level in the serum rises after operative intervention (Nydick *et al.*, Ostrow *et al.*). It therefore follows that the serum activities of the enzymes investigated by us react in the same way after operation and after myocardial infarction. The activities of GOT, LDH, and ALD increase, while those of TRI and CHE decrease.

The serum enzyme levels in various acute infections (including meningitis purulenta, pleural empyema, angina tonsillaris, paratyphoid fever B, and enteritis) are shown in FIGURE 3. Of course, quantitative differences are observed, depending upon the severity of the disease but, in principle, the

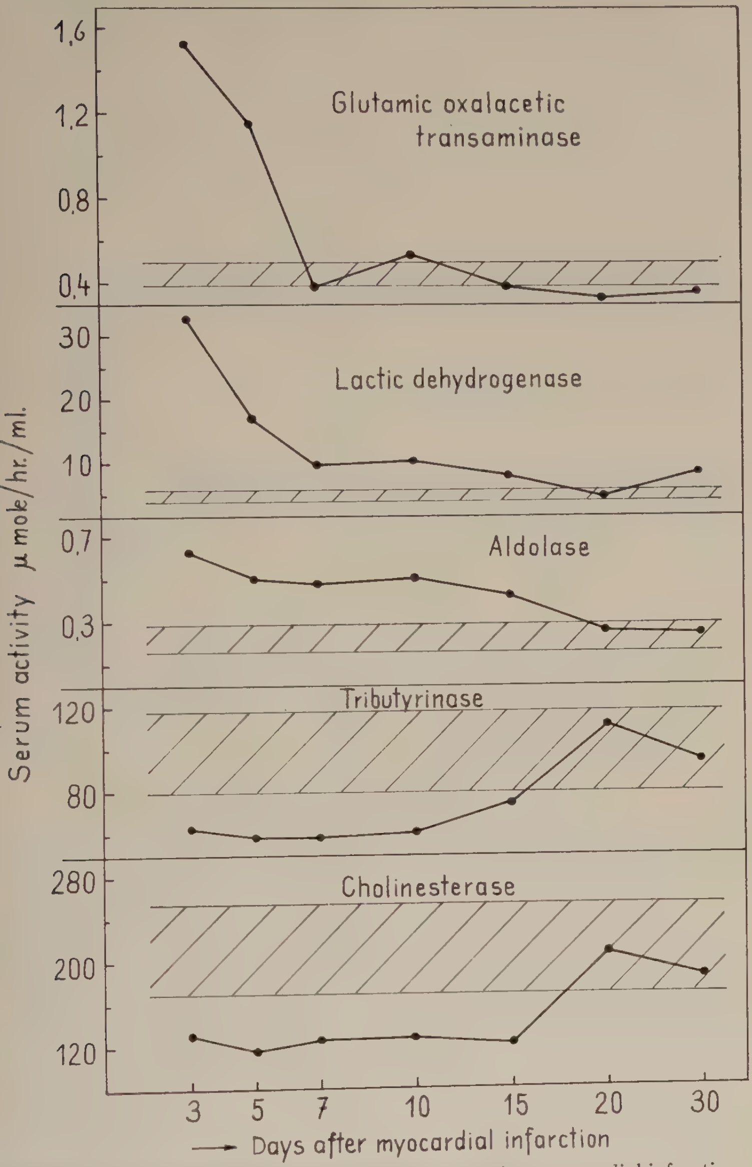

FIGURE 1. Reaction of serum enzyme activities after myocardial infarction.

same reaction is found as in myocardial infarction and operation: a rise in GOT, LDH, and ALD, and a fall in TRI and CHE. Extreme values were obtained in a severe case of lobar pneumonia. In the first days of the disease, we measured the following values (µmole/hr./ml. serum): GOT, 21.16; LDH, 41.2; ALD, 5.4; TRI, 48.5; CHE, 92.0.

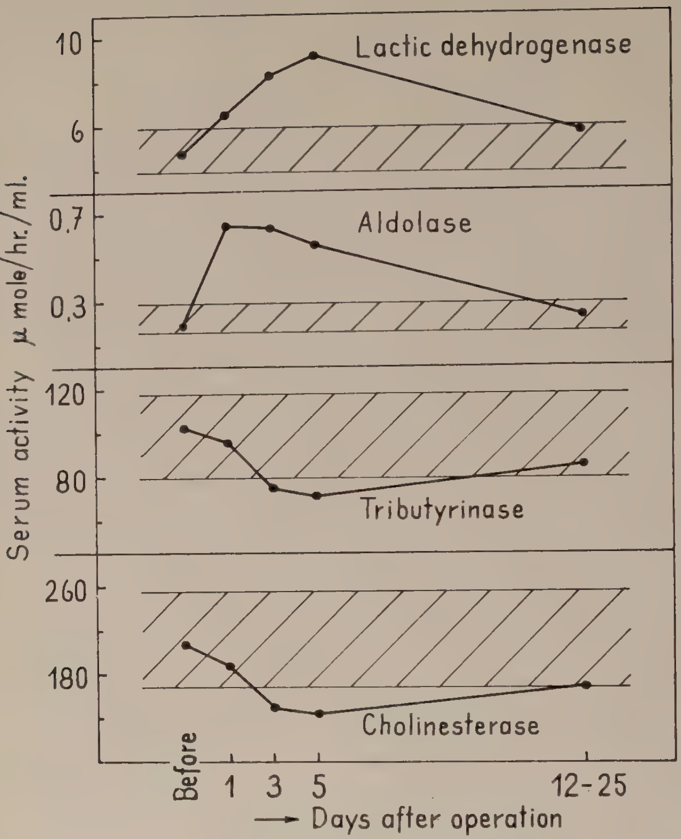

FIGURE 2. Reaction of serum enzyme activities after surgery; mean values for 18 patients.

One of our patients suffering from myocardial infarction acquired a pulmonary embolism after the change in serum enzyme activities brought about by the infarction had returned to normal. As may be seen from FIGURE 4, the myocardial infarction and the pulmonary embolism caused the same change in serum enzyme activities, apart from the absence of ALD increase in myocardial infarction.

Our findings thus demonstrate that myocardial infarction is followed by an increase of GOT, LDH, and ALD, as well as by a decrease of TRI and CHE in serum, and that serum enzymes in cases of surgery, infectious diseases, and pulmonary embolism react in the same manner as in myocardial infarction. It is indeed true that certain quantitative and temporal differences may be observed, depending upon the severity of the complaint; nevertheless, the reaction remains, in principle, the same for all these acute diseases: namely, a rise in GOT, LDH, and ALD and a fall in TRI and CHE. It is nonspecific, since it occurs in a variety of acute diseases.

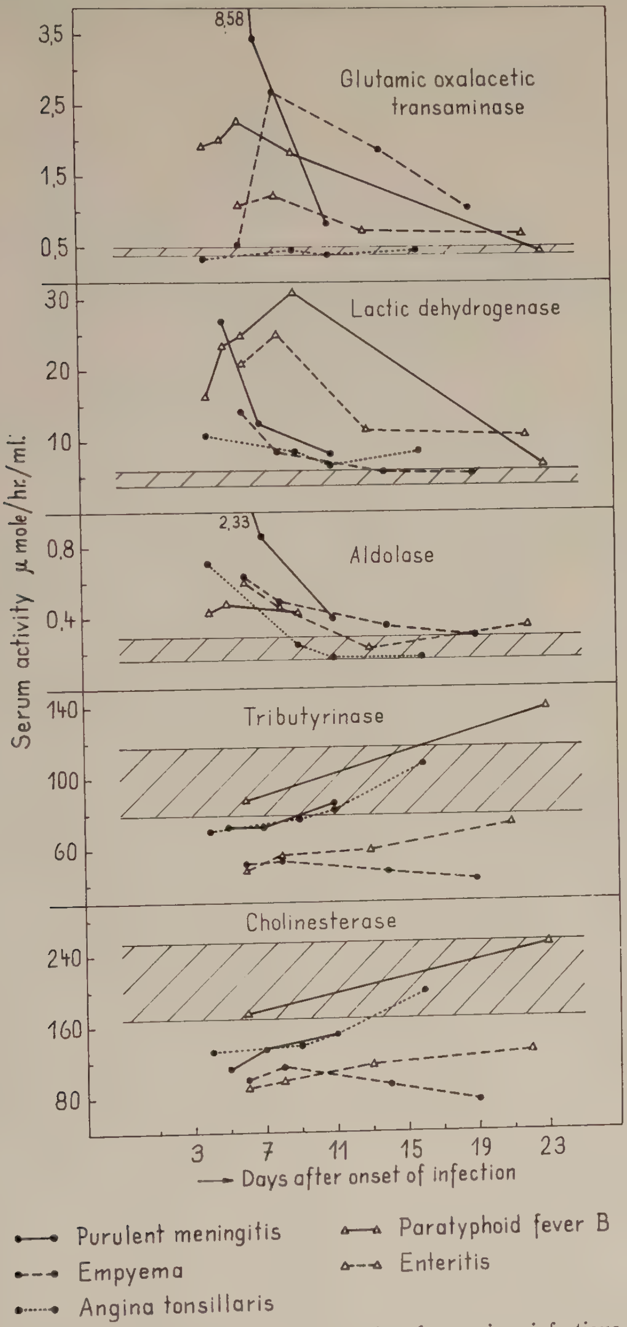

FIGURE 3. Reaction of serum enzyme activities after various infectious diseases.

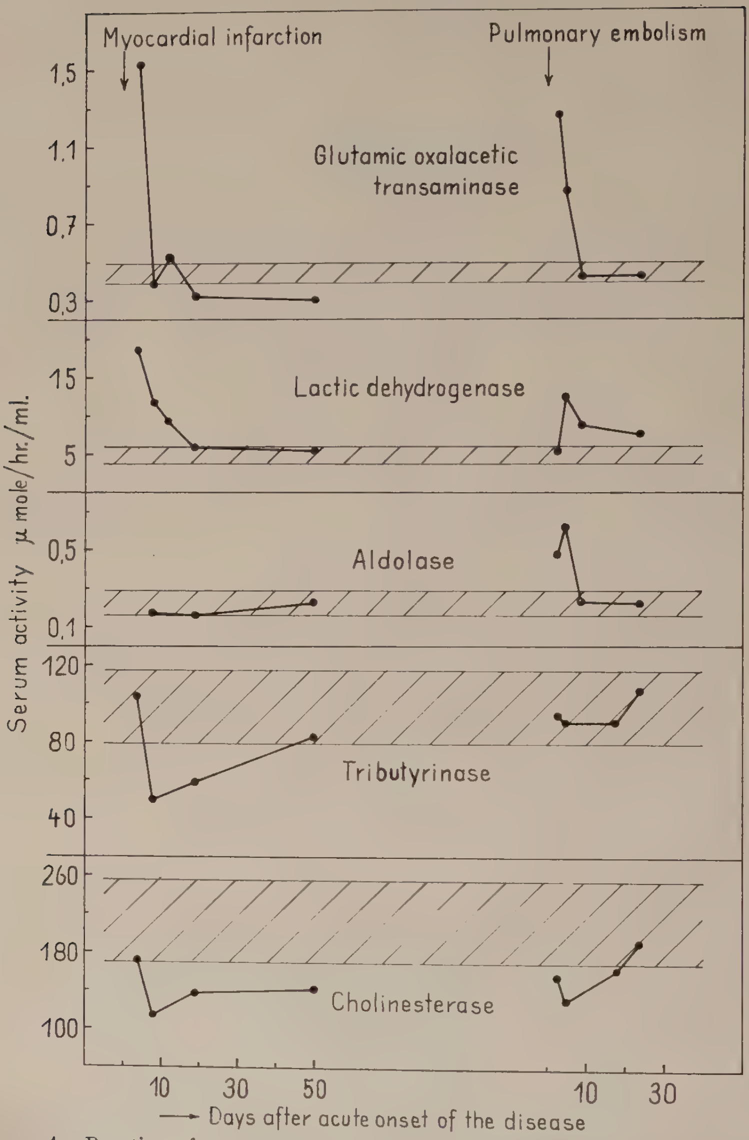

FIGURE 4. Reaction of serum enzyme activities after myocardial infarction and after pulmonary embolism in the same patient.

Other workers (Rudolph *et al.*, Ostrow *et al.*, Ticktin *et al.*, Conrad) also established an increase in GOT following pulmonary, renal, splenic, and mesenteric infarction, acute pancreatitis, crush injuries, burns, hemolytic crisis, and surgery.

Myocardial infarct, operative intervention, infectious diseases, pulmonary embolism, and other acute affections are characterized by a series of further common nonspecific symptoms. In these diseases the organism reacts with rise in temperature, leukocyte increase, changes in hemogram, serum protein variations, increase of blood sedimentation rate, blood sugar and rest nitrogen elevation, and other symptoms which, taken together, we have termed the acute syndrome, the causes of which are very complex. The serum enzyme changes are further symptoms of the acute syndrome.

In our view the reason for the change in serum enzyme activities has not yet been completely elucidated.

Nydick, *et al.* assume that GOT leaks out of the cardiac muscle subsequent to myocardial infarction, thus elevating the level of this enzyme in the serum. This view is based upon the two following findings:

(1) Subsequent to infarction, the GOT disappears from the affected portion of the cardiac muscle, simultaneously reappearing in the serum. It may be demonstrated by experiment on a dog that the GOT content of the cardiac muscle displaying infarction is inversely proportional to the size of the infarction, and that the older the cardiac infarction, the lower the GOT content of the affected cardiac muscle (Nydick *et al.*, Jennings and Wartman).

(2) A rough correlation has been found to exist between the size of the myocardial infarction and the GOT increase in the serum: the larger the infarction, the greater the increase in GOT activity in the serum (Agress *et al.*, Kattus *et al.*, LaDue and Wróblewski).

In our opinion it may well be possible that the elevation in GOT activity in the serum following myocardial infarction is due, at least in part, to leakage from the necrotic cardiac muscle. However, the four following results lead us to believe that enzyme leakage from the cardiac muscle is not the sole factor responsible for the entire observed phenomenon.

(1) In addition to the increase in GOT, LDH, and ALD activity, a fall in TRI and CHE is regularly observed in myocardial infarction, surgery, and acute diseases. It is thus necessary to find an explanation for the phenomenon as a whole that also explains this fall in enzyme activity. This fall cannot be explained by means of the mechanical conception of enzyme leakage from the infarcted heart muscle.

(2) Wróblewski and LaDue injected LDH intravenously into a dog. After injection, the serum level was 10 times the normal value. After 1 hour the greater part of the LDH had already disappeared from the serum. Assuming a degradation or an elimination from the serum at this rate, the LDH content of the infarcted cardiac muscle portion would not suffice to maintain the LDH level in the serum at 3 to 6 times normal value for a period of 5 to 7 days. Human cardiac muscle contains nearly 220,000 units of LDH/gm.; the normal LDH activity in the serum amounts to about 400 units per ml. (LaDue). The destruction of 1 gm. cardiac muscle would cause an LDH activity of 100 units/ml. in 2.2 l. serum. An LDH increase of 1500 units/ml. often observed in myocardial infarction presupposes the destruction of 15 gm. cardiac muscle. Supposing a degradation or an elimination rate of LDH from the serum even much less than 10 times the normal value, a destruction of 15 gm. cardiac muscle per hour would be necessary to maintain a serum level of 1500 units; that is, a destruction of 24 × 15 gm. = 360 gm./day. The elevation of the

serum LDH level to 1500 units for 3 days would require 3 × 360 gm. = 108 gm. heart muscle. A normal human heart weighs about 300 gm.

(3) The enzyme variations occur not only after myocardial infarction, bu also in a variety of acute illnesses in which no cell destruction takes place that is, neither in the heart nor in any other organ (for example, meningiti and angina tonsillaris).

(4) Finally, we (Hauss, Leppelmann, and Plänitz) were able to prove i animal experiments with 265 rats and 31 guinea pigs that, following acute lesion

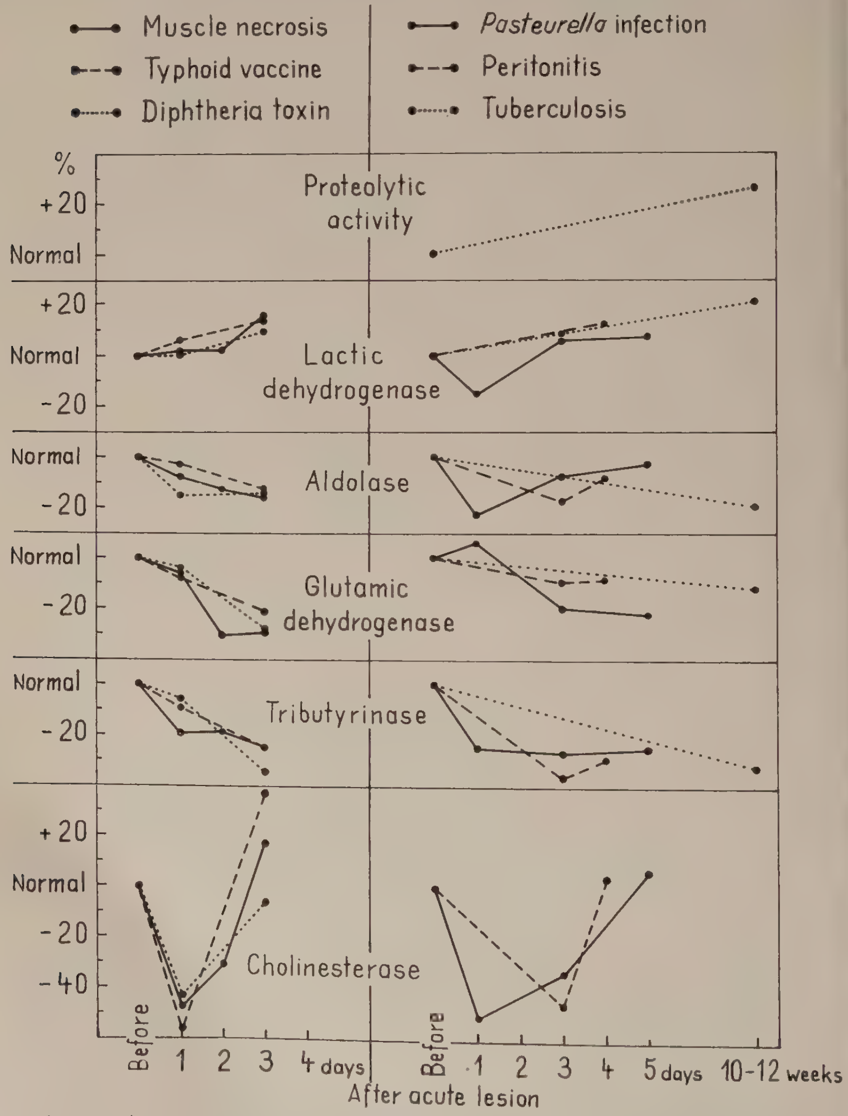

FIGURE 5. Reaction of enzyme activities in the liver after various acute lesions.

extensive enzyme activity changes occur in the liver, an organ of great imporance in enzyme metabolism, at the same time as the change noted in the serum enzyme activities. In a model experiment for myocardial infarction muscle necrosis was induced experimentally in several groups of 15 rats each. Two similar groups were injected with typhoid vaccine and diphtheria toxin respectively, and other rats were infected with *Pasteurella* bacteria. The guinea pigs were treated with tubercle bacilli. A foreign-body peritonitis was produced in further groups of rats by injecting quartz powder into the abdomnal cavity. The rats were sacrificed at intervals of 1 to 5 days following the acute lesion, the guinea pigs 10 to 12 weeks after TB infection.

After sacrifice, we determined the respective activities of LDH, ALD, glutamic dehydrogenase (GDH), TRI, CHE, and also the proteolytic activity (E PROT) in the liver. The experimental results were evaluated by comparing he respective mean values from 15 experimental animals with those from 15 normal animals, the percentage increase or decrease of enzyme activity in the iver after acute lesion then being noted. In these experiments, the *t* distribuion (Student) was also employed as a basis for the statistical calculation. We assumed statistical significance whenever the probability p was lower than 0.05. The results are depicted graphically in FIGURE 5 (for details, see Hauss, Leppelmann, and Plänitz).

This figure shows that the same changes in enzyme activities regularly occur n the liver following various acute afflictions. The same reaction always occurs subsequent to lesion of different types: a rise in LDH activity (in the ase of tuberculosis, also in proteolytic activity); a fall in ALD, GDH, and TRI activity; and a primary decrease in CHE, followed by a secondary rise in CHE.

FIGURE 6 shows the factors influencing the enzyme level in the cell and in he serum. Enzyme degradation and formation in the organ cells, especially

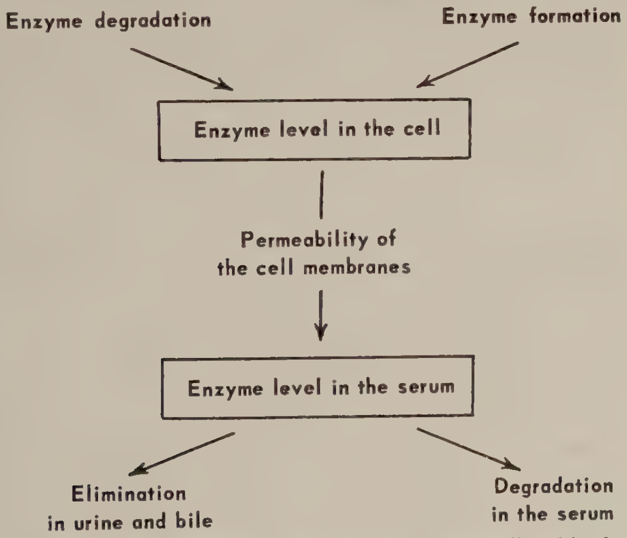
Enzyme degradation
Enzyme formation
Enzyme level in the cell
Permeability of the cell membranes
Enzyme level in the serum
Elimination in urine and bile
Degradation in the serum

FIGURE 6. Factors that influence the enzyme level in the cell and in the serum.

in the liver, and the permeability of the cell membranes are significant for the enzyme level in the cell. The permeability of the cell membranes, the enzyme elimination in urine and bile, and the enzyme degradation in the serum influence the enzyme level in the serum.

We are thus of the opinion that variations of enzyme activity in the serum following myocardial infarction are not to be interpreted solely as consequences of enzyme leakage from the necrotic cardiac muscle cells; these changes are rather to be regarded as a more general phenomenon. A complex reaction of the organism sets in after cardiac infarction, as it does after many other acute diseases. It is this complex reaction that gives rise to a number of symptoms (such as leukocytosis and increase in blood sedimentation rate), and causes the change in enzyme activities. We interpret this reaction as a "*vegetative Gesamtumschaltung*" in Hoff's sense.

Summary

Changes in enzyme activity in human serum set in following myocardial infarction and other acute diseases; thus, glutamic-oxalacetic transaminase, lactic dehydrogenase, and aldolase rise, while tributyrinase and cholinesterase fall. The enzyme activity in rat liver undergoes a uniform reaction after various acute lesions: there is an increase in lactic dehydrogenase activity, a decrease in aldolase, glutamic dehydrogenase, and tributyrinase activities, and a primary fall and secondary rise in cholinesterase.

The enzyme activity changes in the serum due to myocardial infarction and other acute causes are nonspecific; they make their appearance as part of the "acute syndrome" (Hauss). The assumption that the increase in glutamic-oxalacetic transaminase, lactic dehydrogenase, and aldolase in the serum may be explained by leakage of these enzymes from the infarcted cardiac muscle does not appear to us to be a sufficient explanation to account for the phenomenon in its entirety, and especially not for the decrease in tributyrinase and cholinesterase. We prefer to believe that the enzyme activity changes in the serum represent a complex nonspecific reaction on the part of the organism toward a variety of excitations. The changes in enzyme activity observed in the liver of animals subjected to acute lesions serve as a proof of this interpretation.

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CLINICAL AND EXPERIMENTAL STUDIES ON SERUM PYRIDINE NUCLEOTIDE-LINKED DEHYDROGENASES IN LIVER DAMAGE*

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The pyridine nucleotides were discovered and characterized by Otto Warburg,[1] who devised the elegant and sensitive optical methods for measurement of their reduction by specific dehydrogenase systems that have received such wide application in biochemical analysis. In his classic study of the increased serum aldolase activity of tumor-bearing animals, Warburg[2] was the first to show that mammalian blood serum contains a number of glycolytic enzymes including one that uses a pyridine nucleotide as a hydrogen acceptor, namely, lactic dehydrogenase (LD). Since that time two other diphosphopyridine nucleotide (DPN)-linked enzymes (malic[3] and α-glycerophosphate[4] dehydrogenases) have been demonstrated in mammalian extracellular fluids, and changes in serum LD and malic dehydrogenase levels in many disease states have been investigated extensively.[5-10]

Our interest in the oxidizing enzymes of blood serum arose from attempts to study enzymes participating in specific chemical functions of mammary tissue in relationship to carcinoma of the breast. The synthesis of lactose is a biochemical event peculiar to the mammary gland, and there is evidence that lactose is manufactured therein from blood glucose by a series of enzymatic reactions in which uridine diphosphoglycosyl coenzymes are reactants.[11, 12] Attention was directed at first toward uridine diphosphoglucose (UDPG) pyrophosphorylase, which catalyzes the reversible synthesis of UDPG from glucose-1-phosphate and uridine triphosphate. The presence of this enzyme in mammary tissue[13] and in erythrocytes[14] is well established. The UDPG pyrophosphorylase can be measured by coupling with the action of phosphoglucomutase and glucose-6-phosphate dehydrogenase, whereby dihydrotriphosphopyridine nucleotide (TPNH) is formed in stoichiometric amounts for every molecule of UDPG that is split upon the addition of inorganic pyrophosphate. With the aid of preparations of phosphoglucomutase and glucose-6-phosphate dehydrogenase that were devoid of UDPG pyrophosphorylase activity, it was possible to demonstrate small but significant amounts of the latter enzyme in fresh human blood serum (approximately 80 mμmole substrate transformed per 1 ml. of serum per hour at 25° C. (Gotterer and Williams-Ashman, unpublished data). During the course of these experiments it was observed that serum alone catalyzed the reduction of triphosphopyridine nucleotide (TPN) by glucose-6-phosphate. Dehydrogenases specific for TPN had been found in silkworm blood[15] and in the ascitic plasma of mice bearing the Ehrlich ascites tumor,[16] but had not been described in mammalian blood serum Accordingly, a survey of TPN-linked enzymes in serum was undertaken.

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TPN-linked enzymes in serum. Samples of serum without visible evidence of hemolysis obtained from normal adults did not reduce TPN in the absence of added substrates, but catalyzed the formation of TPNH upon the addition of glucose-6-phosphate, 6-phosphogluconate, and isocitrate. Methods for the determination of 6-phosphogluconic (PGD) and isocitric (ICD) dehydrogenases in serum were developed.[17] Magnesium ions and cysteine are necessary for the optimal activity of the former enzyme, and manganous ions for ICD. In normal adults the activity of both enzymes is of the same order of magnitude, that is, in the range of 50 to 250 mμmoles of substrate oxidized per 1 ml. of serum per hour at 25° C. On a molar basis, this represents the same order of activity as that of serum aldolase,[18] glutamic pyruvate transaminase (GPT),[19] and glutamic-oxalacetic transaminase,[20] but is much less than that of LD,[5] malic dehydrogenase,[3] phosphohexoseisomerase,[21] and 5-phosphoriboseisomerase.[22] Serum glucose-6-phosphate dehydrogenase levels are about the same as those of serum PGD.[17] The activity of serum ICD and PGD could not be correlated with age, race, sex, or total serum protein concentration in apparently healthy adults and was within the normal range in the sera of term pregnancies. Serum ICD levels were elevated, however, in the cord blood of newborn infants.

TPNH is barely oxidized by human serum under the conditions of the ICD and PGD test systems when the appropriate substrates are omitted from the reaction mixtures. Furthermore, TPNH was not oxidized by the serum of normal individuals even in the presence of either α-ketoglutarate plus ammonium chloride or of pyruvate plus bicarbonate (saturated with carbon dioxide) and manganous ions, indicating that glutamic dehydrogenase[24] and "malic" enzyme[25] are absent from serum. The stability of TPNH in the presence of serum is in marked contrast to the behavior of dihydrodiphosphopyridine nucleotide (DPNH), which may undergo considerable oxidation when added to serum, expecially if the pyruvate content of the latter is high.*

The rate of reduction of TPN in the ICD and PGD test systems was found to be strictly proportional to the amount of serum added. The ICD and PGD activity of erythrocytes was found to be very much greater than that of serum.[17] However, contact of erythrocytes with serum for many hours at room temperature did not increase the levels of serum ICD and PGD unless hemolysis had occurred.

Serum isocitric dehydrogenase in disease. An examination of the serum ICD activity of 250 patients[26] revealed that the levels of this enzyme seldom fell outside the limits of the normal unless hepatic disease was present. Normal values were observed in subjects with a wide variety of infectious, pulmonary, gastrointestinal, cardiovascular, urological, and neoplastic diseases. Of particular interest was the finding that within the first 24 hours after the onset of symptoms, the levels of serum ICD in patients with large, acute myocardial infarctions were found invariably to fall within the normal range. In this connection it is of interest that the ICD activity of human liver and cardiac muscle, determined from specimens obtained within 10 hours post mortem, were of the same order of magnitude (400,000 to 700,000 mμmoles substrate oxidized per gram of tissue per hour at 25° C.).

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* After this work had been completed, another TPN-specific enzyme, glutathione reductase, was reported to be present in human blood serum by Manso and Wróblewski.[23] These authors observed that the activity of serum glutathione reductase was about ten times as great as serum ICD and PGD.[17] Serum glutathione reductase was found to be elevated in viral hepatitis, although not to such a great extent as serum ICD. Manso and Wróblewski also found abnormally high glutathione reductase levels in the sera of patients with carcinomata. The presence of a very active glutathione reductase in malignant tumors has been reported.[16]

FIGURE 1 shows that within the first 10 days following the onset of jaundice, serum ICD levels were invariably in excess of the upper limit of the normal in patients with acute viral hepatitis. Increases of serum ICD as great as 40 times the normal mean were found in this disease. Large elevations of this serum enzyme were also associated with hepatitis, complicating infectious mononucleosis. Serial determinations of serum ICD in viral hepatitis showed that the levels of this enzyme usually declined to normal around the third week following the onset of icterus. However, when the chronic form of the disease ensued, serum ICD activities remained markedly elevated for as long as 4 months.

In portal cirrhosis, serum ICD was usually within the normal range and was never elevated to values greater than three times the normal mean, even in patients in hepatic coma. In cases of extrahepatic obstruction of the common bile duct, resulting from either benign or malignant causes, serum ICD levels fell invariably within the normal range. In about 50 per cent of patients with objective evidence of liver metastases from malignant disease, the serum ICD activities were greater than the upper limit of the normal. In these situations,

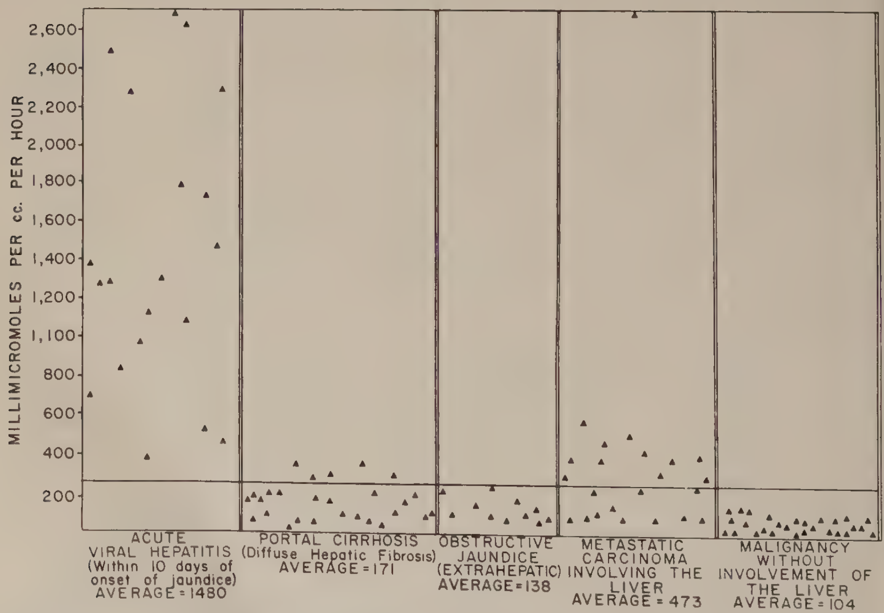

FIGURE 1. Serum isocitric dehydrogenase levels in human liver disease. The horizontal line at the level of 264 represents the upper limit of normal activity for serum ICD.

however, serum ICD was never increased more than six times the normal mean, in marked contrast to the much larger elevations in the activity of this enzyme which were often observed in viral hepatitis. A wide variety of malignant tumors did not cause elevations of serum ICD. If serum ICD is elevated in malignancy, involvement of the liver is indicated.

Serial estimations of serum ICD, GPT, and LD activities in human liver disease[26] showed that alterations in GPT activity were somewhat similar to those of ICD, whereas the changes in LD followed a different pattern and were always of a smaller magnitude than those of ICD and GPT. In viral hepatitis, elevations of serum GPT were sometimes of greater magnitude than those of serum ICD. In patients with the usual benign course of this disease, serum GPT levels tended to remain increased for longer periods of time than those of serum ICD. Moreover, in extrahepatic obstruction, serum ICD activities were never abnormal, whereas those of serum GPT were occasionally elevated, as others[27] have noted. Changes in serum ICD in liver disease bore no relationship to parallel alterations in serum alkaline phosphatase activity and could not be correlated with the extent of hyperbilirubinemia or with the results of flocculation tests.

Serum 6-phosphogluconic dehydrogenase in disease. Studies on a more limited number of patients than were examined for serum ICD activity showed (Wolfson and Williams-Ashman, unpublished data) that serum PGD levels were seldom abnormal in the absence of liver disease. Normal values for PGD were found in seven patients with carcinoma of the breast with extensive osseous metastases and in five patients with carcinoma of the prostate. Serum PGD levels were not increased in cirrhotics. Elevations in serum PGD were observed during the acute phase of viral hepatitis and occasionally in patients with malignant disease with metastases to the liver. The changes in serum PGD in liver disease were never as great as parallel alterations in serum ICD.

Alcohol and polyol dehydrogenase activity of serum. Mammalian liver is known to contain a DPN-linked enzyme, alcohol dehydrogenase (AD), which catalyzes the oxidation of ethanol to acetaldehyde:[28]

$$\mathrm{CH_3CH_2OH + DPN^+ \rightleftarrows CH_3CHO + DPNH + H^+}$$

Another distinct enzyme oxidizes a number of 5-, 6-, and 7-carbon polyols to the corresponding ketose sugars, for example:

$$\text{Sorbitol} + \mathrm{DPN^+} \rightleftarrows \text{D-Fructose} + \mathrm{DPNH + H^+}$$

$$\text{L-Iditol} + \mathrm{DPN^+} \rightleftarrows \text{L-Sorbose} + \mathrm{DPNH + H^+}$$

The activity of this enzyme, polyol dehydrogenase (PD) is greatest in liver and kidney.[29, 30] Neither of these enzymes could be detected in normal human blood serum, as evidenced by the oxidation of DPNH by either acetaldehyde or D-fructose. However, marked activity of both enzymes was found in the sera of certain patients with acute viral hepatitis, or with liver metastases from malignant disease. A typical spectrophotometric determination of these enzymes in the serum of a patient with acute viral hepatitis is depicted in FIGURE 2. In these assay systems, the rate of oxidation of DPNH by either acetalde-

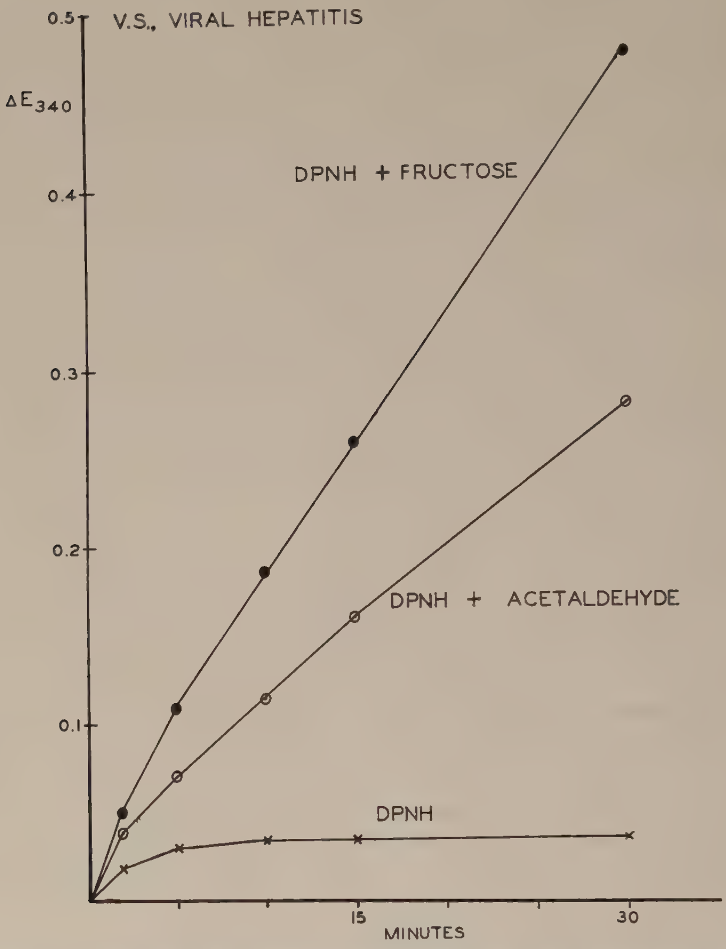

FIGURE 2. Alcohol and polyol dehydrogenase activity of the serum of a patient with acute viral hepatitis. Each vessel contained 100 μmole Tris(hydroxymethyl)aminomethane buffer of pH 7.4; 150 μmole NaCl and 0.3 μmole DPNH in a final volume of 3.0 ml. If added: acetaldehyde 20 μmole; D-fructose 50 μmole. Change in optical density at 340 mμ plotted from the time of addition of serum (0.5 ml.) to the reaction mixture. Temperature 25° C.

hyde of D-fructose was found to be strictly proportional to the amount of serum added. It will be seen from TABLE 1 that the activities of AD and PD in serum bore no constant relationship to one another in patients with viral hepatitis exhibiting the typically increased values of serum ICD. Since neither AD nor PD are present in normal blood serum, the finding of these enzymes in serum is a *qualitative* systemic index of liver disease.

Fate of injected isocitric dehydrogenase. ICD was purified from rat heart by a slight modification of the method of Siebert *et al.*[31] The purified material catalyzed the oxidation of approximately 2000 mμmoles of isocitrate per milli-

TABLE 1

ALCOHOL AND POLYOL DEHYDROGENASE ACTIVITY OF THE SERA OF PATIENTS WITH VIRAL HEPATITIS

Patient	Enzyme activity*		
	Isocitric dehydrogenase	Alcohol dehydrogenase	Polyol dehydrogenase
1	3400	493	384
2	768	133	12
3	390	134	260
4	3480	445	778
5	4500	822	480

* Mμmoles substrate oxidized or reduced per 1 ml. serum per hour at 25°C. Isocitric dehydrogenase determined by the method of Wolfson and Williams-Ashman.[17] Alcohol and polyol dehydrogenases determined under the conditions shown in FIGURE 2.

gram of protein per minute under the conditions of the ICD assay system.[17] The preparation was devoid of glutamic dehydrogenase activity, as evidenced by its inability to catalyze the oxidation of DPNH upon the addition of α-ketoglutarate and ammonium chloride at *p*H 7.4. Just before administration to experimental animals, the purified enzyme was dialyzed thoroughly against 0.15 M NaCl at 2° C. The enzyme was injected into the saphenous vein of rats weighing approximately 300 gm. under ether anesthesia. Sufficient amounts of enzyme were injected to raise the serum ICD activities thirtyfold to seventyfold within 2 minutes after injection. Blood samples were removed at various time intervals from the jugular vein. From semilogarithmic plots of serum ICD activity versus time, the mean half life of the injected enzyme was found to be 60 min. No difference in the rate of disappearance of injected ICD from the blood stream was observed between male and female animals of the same age. Rat blood serum incubated with purified ICD at 37° C. inactivated the enzyme at very slow rates; more than 50 per cent of the activity remained after 24 hours.

The rapid disappearance of activity after the injection of enzymes directly into the circulation has been noted by Warburg[2] for aldolase and by Huggins[32] for acid phosphatase. It would appear that the maintenance of greatly increased serum ICD levels following acute injury to previously healthy hepatic cells (as, for instance, in acute hepatitis) must entail a continual outflow of this enzyme from dead or damaged cells into the blood stream.

Carbon tetrachloride poisoning. Soon after the administration of carbon tetrachloride, the liver parenchyma undergoes an inflammatory reaction which is usually followed by massive necrosis. The hepatotoxic action of carbon tetrachloride appears to be related to a disruption of mitochondrial structure by this agent.[33] The hepatic lesions produced by carbon tetrachloride bear considerable resemblance to those resulting from hepatitis of viral origin. Large increases in the activity of some, but not all, enzymes in blood serum have been reported to follow the administration of carbon tetrachloride to experimental animals. For instance, the careful studies of Bruns and Neuhaus[34] demonstrated that the aldolase and phosphohexoseisomerase activity of mouse serum rose enormously in animals poisoned by carbon tetrachloride, while the levels of these enzymes in liver fell dramatically; under the same experimental conditions, serum tributyrinase, amylase, and alkaline phosphatase were barely affected. In order to gain some insight into the factors which govern the efflux of enzymes into the circulation following liver damage, the serum levels of ICD, PGD, LD, AD, and PD were studied at various time intervals after administration of carbon tetrachloride. All of these enzymes appear to be confined mainly to the soluble fraction of liver cells, that is, they are not bound to either mitochondrial or microsomal structures, and all of them use a pyridine nucleotide as a coenzyme. The carbon tetrachloride was dissolved in mineral oil and given in a single dose (5 cc. CCl_4/kg.) by stomach tube to Sprague-Dawley rats weighing between 350 and 400 gm. Blood samples were taken from the abdominal aorta under ether anesthesia. It will be seen from FIGURE 3 that serum ICD activity rose as much as four hundredfold after the injection of this poison. In the same animals, marked AD and PD activity appeared in the sera of the animals which had received carbon tetrachloride. The change in activity of all of these enzymes occurred more rapidly in female than in male rats of the same age. Serum PGD levels increased after the administration of carbon tetrachloride, but to a much lesser extent than those of serum ICD; serum LD was altered only to a very small extent.

Determinations were also made of the activities of AD and PD in the livers of these animals. In order to minimize both the destruction of pyridine nucleotides and the oxidation of DPNH by other enzymatic pathways, the nuclear and mitochondrial fractions were first removed. The livers were homogenized in 0.25 M sucrose, centrifuged at 600 g for 5 min., and the supernatant fluid again centrifuged at 14,000 g for 30 min. All operations were carried out at 2° C., and the final supernatant fluid was used for measurement of the liver enzyme levels. Five hours after the administration of carbon tetrachloride, the liver levels of AD and PD were within the limits of the normal whereas by 18 hours they had dropped to about 60 per cent of normal in female, but not in male animals. At this time interval, the serum levels of these enzymes had risen to a maximum in female, but not in male rats (FIGURE 3). By 48 hours, at which time the serum AD and PD activities had fallen considerably, the liver levels of both enzymes were only about 40 per cent of the normal in both sexes. In the livers of untreated animals, the activities of AD (120,000 to 180,000 $m\mu$moles substrate oxidized per gram of tissue per hour at 25° C.) and PD (200,000 to 250,000 units) were about the same in male and female rats and were of the same order of magnitude as those of PGD (100,000 to 200,000 units). The activities of rat liver ICD (900,000 to 1,200,000 units) and LD (14,000,000 to 18,000,000 units) were much greater than those of AD, PD, and PGD in normal animals. Clearly the changes in the levels of these pyridine nucleotide-linked dehydrogenases in liver and in serum, which attend the administration of carbon tetrachloride, bear no direct relationship to the normal activities of these enzymes in either liver or in serum.

Other types of experimental liver damage. Twenty-four hours after 70 per cent partial hepatectomy, the serum ICD of male rats was thrice that of sham-operated animals. After complete ligation of the common bile duct of either male or female rats, moderate elevations (up to eightfold) of serum ICD were observed 24 and 48 hours after surgery, whereas serum PGD was barely altered under the same conditions. This procedure led to the appearance of PD activity in serum. The changes in serum ICD and PD were considerably smaller after partial than after complete ligation of the bile ducts. Serum ICD was within the normal range 24 hours after the severe stress of limb ligation shock.

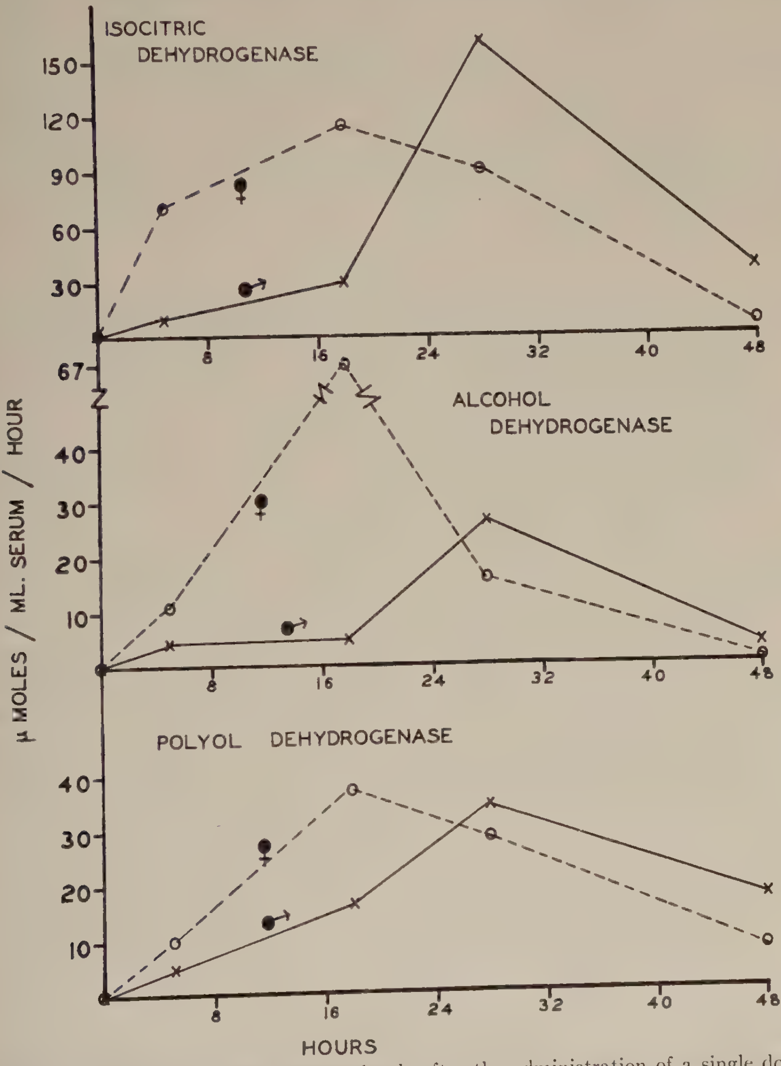

FIGURE 3. Changes in serum enzyme levels after the administration of a single dose of carbon tetrachloride to the rat. Each point represents the mean enzyme level determined from a group of four animals.

Summary

In addition to a number of DPN-linked dehydrogenases, human and rodent sera have been shown to contain certain oxidizing enzymes which use TPN as hydrogen acceptors. Large increases in serum isocitric dehydrogenase accompany viral hepatitis in man. The levels of this enzyme were often elevated in malignancies with metastases to the liver, but were seldom changed in portal cirrhosis and in extrahepatic obstruction of the common bile duct. The changes in serum 6-phosphogluconic dehydrogenase in human liver disease were much smaller than corresponding alterations in serum isocitric dehydrogenase. The activity of the latter enzyme was within the limits of the normal in a wide variety of other disease states. Acute viral hepatitis in man leads to the appearance of alcohol dehydrogenase and polyol dehydrogenase activity in blood serum. Alterations in the serum levels of these pyridine nucleotide-linked dehydrogenases after various types of experimental liver damage in the rat are discussed.

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TRANSAMINASE ACTIVITY IN SUSPENSIONS OF PARENCHYMATOUS LIVER CELLS*

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When measured in serum, glutamic pyruvic transaminase (GPT) is a more sensitive index of liver injury than glutamic-oxalacetic transaminase (GOT).[1] The mechanism of the elevation of these serum enzyme activities has remained obscure. Biochemical determinations on liver biopsies may be expected to help in elucidating this mechanism, provided that a satisfactory basis of reference for such measurements can be found. As most of the metabolic functions of the liver are believed to be in the parenchymatous cell, this structure would be the most logical standard of reference if it could be obtained in suspension, capable of being counted separately, morphologically and enzymatically intact, reasonably free from admixture with other tissue components such as Kupffer cells, and in a yield sufficiently high to be representative of the parenchymatous cell population as a whole.

Slices about 1 cm. in thickness were cut from the freshly excised organ of the rat, incubated at 0° C. for 12 to 16 hours in calcium-free Lockes solution, containing 21.8 gm. of sodium citrate per liter. The tissue was then gently pressed through stainless steel sieves, centrifuged at 200 g for 8 min., and washed once by resuspending the precipitate in fresh medium and repeating the centrifugation. The cellular yield was calculated from a count of nuclei in quadruplicate on the final cell suspension and by determining the number of intact parenchymatous cells per 2000 to 4000 nuclei on stained smears.

We have obtained an average of 42,000 parenchymatous cells per mg. wet weight of liver, almost free from Kupffer cells, and intact to microscopy and phase contrast microscopy. The cells were assayed for their contents of GOT and GPT by a method[2] in which the tissue homogenate was allowed to act on nonlimiting concentrations of buffered substrates for 20 min. The reaction was stopped by the addition of phosphoric acid and digestion of the enzyme with pepsin. After 30 min. the *p*H was restored by the addition of sodium hydroxide. Oxalacetate formed in the GOT reaction was decarboxylated to pyruvate at 60° C., and the final product of the reaction (pyruvate) was assayed with lactic acid dehydrogenase and reduced coenzyme I. Blanks were prepared by tipping the enzyme into the substrate acid-pepsin mixture. All determinations were carried out in duplicate.

On an average, 85 per cent of the expected value of these enzymes was recovered from the cells, supernatants, and washings. In the cell suspension the mean recovery of GOT was 95 per cent, that of GPT only 26 per cent of the expected value, based on a parenchymatous cell population of 120,000 per mg. wet weight.[3] Balance studies indicated that GPT leaked out of the tissue at

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TABLE 1

DISTRIBUTION OF GOT AND GPT IN PREPARATION OF PARENCHYMATOUS LIVER CELLS

	Liver No. F 4			Liver No. F 6		
	Distribution		GOT/GPT	Distribution		GOT/GPT
	% GOT	% GPT		% GOT	% GPT	
Whole tissue			3.8			3.9
Overnight medium	2.0	5.4	1.3	3.2	5.9	1.5
Supernatant 1	51.5	79.7	2.2	67.6	83.4	2.3
Supernatant 2	0.4	1.4	0.9	1.8	1.7	3.0
Cell suspension	46.1	13.4	11.8	25.8	8.7	8.3
On sieve	—	—	—	1.5	3.0	13.9

the stage of breakup into the component cells, that is to say, into the first supernatant (TABLE 1). Media composed of sucrose and ethylenediaminetetraacetic acid disodium salt (Versene) in varying proportions resulted in a similar loss of GPT from the cells.

The method of cell separation, designed to produce a yield of cells sufficiently high to be representative, involved a number of unphysiological procedures: (1) the tissue was removed from the animal 12 to 16 hours before preparation of the cell suspension; (2) it was chilled to 0° C.; (3) it was possibly subject to anoxia during the incubation period; (4) citrate may have been metabolized during the incubation period; and (5) further cellular injury may have occurred as the organization of the tissue was destroyed, that is, in the process of separation.

In view of the possible analogy of this preferential loss of GPT to clinical situations involving elevations of serum enzymes, further study of this phenomenon in a system free from the above-mentioned defects appeared to be of interest.

The rat was anesthetized in a room kept at 37° C. and the right lobes of the liver were excised. Slices 0.2 to 0.4 mm. in thickness were cut immediately, rinsed, weighed, and vigorously shaken in the suspending medium. The slices, the medium, and a precipitate formed from the medium after centrifugation, consisting mainly of red cells, were examined immediately for their contents of GOT and GPT. Recoveries were based on transaminase activities determined on whole-tissue homogenates prepared from the same lobe of liver. Preliminary experiments showed that an appreciable amount of enzyme would be lost from the slices after an hour of shaking, and that a tonicity of about 190 mEq./l. was desirable. Total recovery of enzymes from the system was 110 ± 14 per cent for GOT and 85 ± 9 per cent for GPT. When a Krebs-Ringer phosphate buffer at *p*H 6, 7, and 8 was used, similar amounts of enzymes remained in the slices at the end of the hour (TABLE 2). Phosphate is a poor buffer at *p*H 9, and a carbonate bicarbonate buffer was therefore used. GPT was lost from the slices in considerable excess over GOT at this *p*H whether the bicarbonate was used alone or with phosphate (TABLE 2). On the other hand, if the experiment was carried out in an atmosphere of 5 per cent carbon

TABLE 2

EFFECT OF COMPOSITION OF MEDIUM ON GOT, GPT, AND PROTEIN CONTENT OF RAT LIVER SLICES

Medium	*p*H	Liver slices GOT	GPT	Protein	Protein recoveries
		%	%	%	%
Krebs-Ringer phosphate	6.0	91	85	84	83
Krebs-Ringer phosphate	7.0	75	84	87	88
Krebs-Ringer phosphate	8.0	77	78	71	110
Krebs-Ringer bicarbonate	9.0	75	59	87	89
Krebs-Ringer phosphate with bicarbonate	9.0	72	8	88	82
Krebs-Ringer bicarbonate (5% CO_2 and air)	7.5	80	74	81	85
Krebs-Ringer phosphate	9.0	71	45	65	85
Krebs-Ringer bicarbonate (2 parts in 10 0.1 M alanine)	9.0	76	79	72	111
Krebs-Ringer bicarbonate (2 parts in 10 0.1 M alanine)	9.0	78	78	73	103

dioxide and air, thus keeping the bicarbonate buffered medium at *p*H 7.5, the amounts of enzymes lost from the cells are again of comparable magnitude (TABLE 2). The suggestion that the leak of GPT is an effect of *p*H rather than bicarbonate is further borne out by the fact that, if a phosphate medium is used and steps are taken to keep the pH at 9 by the addition of sodium hydroxide, the loss of GPT from the slices again exceeds the loss of GOT (TABLE 2). It therefore appears that one of the factors leading to a loss of GPT is *p*H.

To investigate this further, alanine, which is both a good buffer at *p*H 9 and a substrate for GPT, has been used. If alanine is added to the bicarbonate buffer at *p*H 9 the effect at this *p*H noted in phosphate and bicarbonate is eliminated.

In the preparation of liver slices a certain amount of tissue is damaged mechanically, and injured cells may be expected to release their protein into the medium. At *p*H 9, where in most instances the loss of GPT from the slices is much greater than that of GOT, there is no corresponding loss of protein.

From these data it appears that the behavior of these enzymes with respect to tissue injury is very different. It remains to be determined whether the difference in release is merely one of rate, or whether the presence of GOT in the medium can be accounted for largely by loss from cells injured in the preparation of the slices.

The retention of GPT in the cells in the presence of alanine at *p*H 9 is of particular interest. It has been shown by Rowsell[4] that GPT in liver is a component of an important deamination mechanism for a number of amino acids. The concentration of GPT in liver has been shown to vary directly with the protein catabolism of the organism.[5] Therefore, in a sense GPT is an adaptive enzyme. The fact that the leakage of this enzyme is prevented by one member of its substrate pair may give a clue to one of the mechanisms that control enzyme activities within a tissue.

In summary, GPT is lost from liver cells and liver slices under certain conditions more readily than GOT or protein. At *p*H 9 this effect can be demonstrated in the presence of phosphate or bicarbonate, but not in the presence of alanine. The system may be of further use in the *in vitro* study of liver injury.

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Discussion of the Paper

H. J. Leppelmann (*University of Münster, Münster, Germany*): With reference to the probable mechanism of serum enzyme elevations in liver diseases, I should like to offer a short calculation. U. Gerlach, in our laboratory, measured the activity of sorbit dehydrogenase in human serum and liver. He found none of this substance in the sera of normal humans, but there was about 200 units/gm. wet weight in the liver of such individuals. In cases of viral hepatitis he found from 150 to 200 units/ml. in the sera; that is, with an assumed serum level of 175 units/ml., 2500 ml. serum × 175 units equals 437,000 units of sorbit dehydrogenase in whole serum. This means that the destruction of about 2180 gm. of liver would be required to cause the elevation of serum sorbit dehydrogenase to increase to a level of 175 units/ml.; however, a normal human liver weighs only about 1500 gm. This calculation shows that enzyme leakage from the liver cannot be solely responsible for the elevation of the serum sorbit dehydrogenase; an active secretion of this enzyme appears to be indicated. No elimination or degradation of the enzyme has been considered in this calculation.

Keith S. Henley: I am sure Leppelmann would be the first to agree with me that calculations of the kind that he has made are somewhat difficult to interpret. In the rat, the activities of GOT and GPT are such that these enzymes could produce the liver's own weight of oxalacetate in 20 min. or of pyruvate in 2 hours. Unfortunately, we are obliged to estimate these enzymes under conditions of maximum activities. This means using thoroughly unphysiological concentrations of substrates, so that any extrapolation of data from the test tube to the intact organ is very hazardous indeed.

ENZYMATIC DEFICIENCY IN CONGENITAL GALACTOSEMIA AND ITS HETEROZYGOUS CARRIERS

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Congenital galactosemia is an abnormality in humans that has the four following characteristics: (1) it is an inborn error and undoubtedly of hereditary origin; (2) it is a metabolic abnormality affecting galactose metabolism; (3) if particular precautions are not taken, the abnormality manifests itself as a serious disease that is fatal or leads to permanent damage to tissues as manifested by cataracts, liver cirrhosis, and feeble-mindedness; and (4) if the newborn infant is kept on a diet completely free of galactose, disease symptoms do not develop. However, as will be described in this report, the abnormality persists and is independent of whether galactose is administered.

For many years this biochemical abnormality was largely unknown with the exception of one outstanding feature; namely, that the administration of lactose and galactose brought about galactosemia and galactosuria. In 1956 Schwarz *et al.*[1] made the significant additional observation that the administration of galactose to galactosemic subjects gave rise to an accumulation of α-galactose-1-phosphate in the red blood cells. This observation ruled out a galactokinase defect as a basis for galactosemia. However, it was still uncertain whether another enzymatic defect might be an underlying cause for this phenomenon, or whether it was a more complicated disturbance of the regulation of galactose metabolism. Schwarz *et al.*[1] and previous authors often cited a defect in galacto-Waldenase* as the most likely cause of both clinical and biochemical symptoms. Such a defect, however, would be inconsistent with the fact that the administration of a galactose-free diet in the newborn not only prevents the specific symptoms of the disease, but also permits the normal growth and development of the child. The latter fact should be emphasized, since it is known that the deposition of galactose in the central nervous system as galactolipids is a process that takes place exclusively after birth.[2] Under existing concepts of galactose metabolism, a block in the galacto-Waldenase would not only eliminate the use of galactose for these purposes, but also prevent the use of glucose as a source of carbon for the synthesis of complex galactolipids in the central nervous system.

When Leloir in 1951 discovered uridine diphosphoglucose (UDP) and the galacto-Waldenase,[3] that is, the enzyme that catalyzed the reaction UDP glucose $\rightleftharpoons$ UDP galactose, he also predicted that there must be another and independent enzyme that incorporates the galactose into uridine nucleotide. This enzyme was described a few years later in yeast adapted to galactose[4] as well as in mammalian liver.[5] Since the name "galacto-Waldenase" has been used in different contexts, it was proposed, on the basis of some further studies on the UDP-galactose (UDPG) enzymes, to introduce a new terminology.[6]

* Waldenases = the uridine diphosphate galactose epimerase.

The enzyme that catalyzes the incorporation of galactose-1-phosphate (P-Gal) into UDP-glucose was called P-Gal uridyl transferase, and it catalyzes the following reaction:

$$\text{P-Gal} + \text{UDPG} \rightleftharpoons \text{PG} + \text{UDPGal} \tag{1}$$

The inversion enzyme proper catalyzes the above mentioned reaction; namely,

$$\text{UDPG} \rightleftharpoons \text{UDPGal} \tag{2}$$

For various reasons it was found more likely that if the case of congenital galactosemia really represented an abnormality based on an enzyme defect, the enzyme involved would be PGal uridyl transferase and not the inversion enzyme. The studies that one of us (H. M. K.) did in collaboration with E. P. Anderson and K. J. Isselbacher[7] fully confirmed this assumption and showed, moreover, that enzymes catalyzing the other steps in what one might call the "Leloir pathway" are not affected;[8] this is illustrated in TABLE 1. The same defect could be demonstrated in blood from the umbilical cord of the newborn[9] (TABLE 2). This is a direct demonstration that the enzymatic defect is full-fledged at birth and is independent of the exposure of the cells of the body to the high levels of galactose that occur as the infant is offered milk.

Although it was fortunate that the studies of the enzymes of the Leloir pathway could be followed in hemolyzates of red blood cells, human red blood cells

TABLE 1

ENZYMES OF GALACTOSE METABOLISM IN HUMAN HEMOLYZATES
(Activity Expressed in μMoles of Substrate Consumed per Hour per Milliliter of Packed-Cell Hemolyzate)

Condition of subjects	Galactokinase		P-Gal-uridyl transferase		Galacto-Waldenase		PP uridyl transferase	
	Subjects	Activity	Subjects	Activity	Subjects	Activity	Subjects	Activity
	No.	*Avg.*	*No.*	*Avg.*	*No.*	*Avg.*	*No.*	*Avg.*
Normal	3	0.10	15*	0.82	3	0.32	9	1.20
Galactosemic	3	0.08	10	0.02	3	0.35	8	1.85

* This figure also includes infants that were on galactose-free diets.

TABLE 2

GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE IN HEMOLYZATES FROM CORD BLOOD
(Activity Expressed in μMoles of Substrate Consumed per Hour per Milliliter of Packed-Cell Hemolyzate)

Subject	Enzymatic activity
Normal adult	1.0 to 3.0
Normal infant (cord blood)	2.0
Galactosemic adult	<0.02
Galactosemic infant (cord blood)	<0.02

TABLE 3

URIDYL TRANSFERASES IN HUMAN LIVER HOMOGENATES

	μMoles incorporated into nucleotide per gm. liver per hour	
	Gal-1-P*	*G-1-P**
Nongalactosemic adult*	>15.0	
Nongalactosemic infant*	>25.00	>4.0
Galactosemic adult†	1.2	
Galactosemic infant†	<0.3	>3.0

* Post-mortem
† Biopsy

are nonnucleated, and one might argue that such cells are not as representative of over-all metabolism as the liver, which normally carries the burden of converting orally administered galactose into glucose. It was desirable, therefore, to ascertain whether or not the same enzymatic defect could be demonstrated in liver cells. TABLE 3 illustrates the results of experiments that were carried out with C^{14}-labeled galactose obtained from Horace Isbell of the National Bureau of Standards, Washington, D. C. The three following points should be noted. (1) The nongalactosemic samples were obtained post-mortem; undoubtedly, therefore, they represent minimum values. (2) The enzyme UDPG pyrophosphorylase, which catalyzes incorporation of pyrophosphate (that is, $PP + UrPG \rightleftharpoons PG + UrPP$), is present in all the specimens. For various reasons the quantitative estimation gives values that are too low. (3) The P-Gal uridyl transferase is completely absent in one case of galactosemia or, to be more nearly correct, is nondetectable. The other case of galactosemia involved a 24-year-old subject; his liver tissue showed some activity, although greatly lowered (compare TABLE 3). In this case we must admit that our method does not permit us to distinguish between an incomplete defect and the development, on a modest scale, of an adaptative alternative but closely related pathway such as that recently described by various authors.[10, 11] The fact that the same subject was able to form glucuronide from administered radioactive galactose[12] can be explained as well on either basis.

Holzel and Komrower[13] have initiated family studies on congenital galactosemia. These studies were performed by means of galactose tolerance tests done on relatives of subjects affected with congenital galactosemia. The outcome of these studies showed one particularly striking feature: that a lowered tolerance for galactose was encountered in siblings as well as in parents of galactosemic subjects. On testing in the latter case, however, as a rule only one of the parents showed lowered tolerance, for galactose, whereas the other showed normal tolerance. Such an observation is at variance with what is observed in the heterozygous-state of diseases inherited on the basis of a so-called single recessive gene.

Since galactose tolerance tests are time consuming, tedious, and not particularly accurate or specific, we hoped that it would be possible, by developing enzymatic methods that would provide the opportunity to follow zero-order kinetics, to circumvent the above-mentioned difficulties in an extended study

TABLE 4

GAL-1-P URIDYL TRANSFERASE ASSAY ON HEMOLYZATES FROM NORMAL PERSONS

	Age	Avg. activity, μl./hr. O_2 uptake for 0.3 cc. hemolyzate
Male, N. K.	30 yrs.	40.0
Male, H. K.	49 yrs.	33.7
Female, R. T.	40 yrs.	29.5
Female, N. S.	56 yrs.	31.2
Female, N. M.	31 yrs.	29.9
Female, E. Y.	32 yrs.	30.6
Male, P. S.	33 yrs.	26.7
Female, J. H.	22 yrs.	29.8
Male, L. A.	—	28.7
Female, N. A.	—	31.3

TABLE 5

GAL-1-P URIDYL TRANSFERASE ASSAY ON HEMOLYZATES FROM FAMILIES OF GALACTOSEMIC CHILDREN

	Age	Avg. activity, μl. O_2 uptake/hr. for 0.3 ml. hemolysate
Galactosemic (P.O.)	2½ yrs.	1.5
Unaffected brother (S.O.)	10 mos.	33.1
Mother	30 yrs.	27.8
Father	35 yrs.	*26.1*
Galactosemic (K.C.)	4½ mos.	4.8*
Unaffected sister (S.C.)	6 yrs.	30.0
Mother	26 yrs.	28.7
Father	32 yrs.	27.5
Maternal grandmother	45 yrs.	28.0
Maternal grandfather	61 yrs.	34.0
Mother (P.)	31 yrs.	*16.7*
Father (P.)	36 yrs.	*20.8*
Galactosemic (A.F.)	3 mos.	0.9
Unaffected sister (L.F.)	3 yrs.	37.0
Unaffected sister (M.F.)	2 yrs.	39.1
Mother	—	*22.2*
Father	—	*16.6*

* Received 120 ml. whole blood by transfusion 5 weeks prior to assay.

of the genetics of this disease. Conditions for following zero-order kinetics of the P-Gal uridyl transferase in hemolyzates were provided by techniques that will be described in detail elsewhere by one of us (H. N. K.). The principal outcome of these studies is summarized in TABLES 4 and 5. To date, blood from 14 members of 4 independent families has been investigated by this method. Although early in the project no greatly reduced activity was found in relatives, it has become apparent as the study has progressed that some, but not all, parents do have a significant lowering in activity of this enzyme. Sometimes both parents have such lowering. All parents in TABLE 5 have levels below the mean for the normals. Values for relatives greater than one and one-half standard deviations below the mean for normals are italicized.

Thus, the galactose tolerance tests of Holzel and Komrower,[13] as well as an assay for the enzyme itself, in hemolyzates suggest that some parents and relatives of galactosemic children have an impairment in galactose metabolism while others have none. This difference between parents is puzzling. The presence of a suppressor gene or the existence of two or more forms of galactosemia might explain these differences. However, a simpler explanation may be merely that heterozygous individuals, as is true of normal persons, have moderate variations in amounts of enzyme. This may give rise to a single-modal distribution curve that overlaps, on the high side, the distribution curve for normals.

We are of the opinion that these findings will provide the basis for further investigation into the interaction of genes on enzymes and the reflection of this interaction on the status of enzymes in erythrocytes.

The advantages, if any, of using young erythrocytes[14, 15] in this assay are under investigation in our laboratory.

Acknowledgment

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HEREDITARY ENZYMATIC EFFECTS AS ILLUSTRATED BY HYPOPHOSPHATASIA*

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Many conditions have been recognized and categorized as hereditary diseases; these are particularly interesting to the biochemist and clinician, since they are usually associated with an anomaly in a metabolic pathway. With advances in biochemical knowledge and methods, details of these diseases have been investigated, elucidated, and reviewed extensively.[1-7] It is obvious that countless conditions resulting from gene action are so mild as to escape clinical notice. In addition, results of gene action are often of a quantitative nature, so that only marked deviations can be readily discerned. For example, a person of nearly normal intelligence who has phenylpyruvic oligophrenia, a disease with which mental retardation is usually associated, will frequently go unnoticed.[8]

FIGURE 1 illustrates an idealized enzymatic reaction that can be applied to this discussion. Ordinarily substrate *A* is converted to product *B*. If the enzyme is deficient or absent, then less of *B* will be formed and, dependent upon quantitative relationships, *A* will accumulate. Lack of *B* may result in disease, as in glycogen storage disease; accumulation of *A* might be pathological, as with galactose in galactosemia. Finally, as is suspected in phenylpyruvic oligophrenia, the substrate may be chemically converted to toxic side products and thus result in disease. If *A* were a pool of protein precursors and *B* a protein, then an abnormal protein *C* could form, as in sickle-cell anemia, or a lack of protein formation might become evident, as in the coagulation defects. All hereditary disorders of incomplete metabolism portray a similar sequence of biochemical circumstances, but often it is difficult to connect accumulated substance with disease.

Much of the investigative effort is directed toward an identification of the specific enzyme defect. TABLE 1 shows the hereditary diseases in which the specific enzyme defect has been demonstrated. In each case the activities of these enzymes are deficient in the indicated tissue. The possibility exists that the enzyme is present, but inactive because of a slight abnormality in protein structure. Most determinations have been made with liver; only three diseases have been identified by the use of blood.

Two of these conditions are discussed elsewhere in this monograph: galactosemia (Kirkman and Kalckar) and the hemolytic disease resulting from poisoning by primaquine or naphthalene (Gross[10]). The third disease, hypophosphatasia, is an example of an hereditary disease in which the associated enzymatic deficiency has been demonstrated in serum, liver, bone, kidney, and intestine. This report demonstrates the deficiency in activity in white blood

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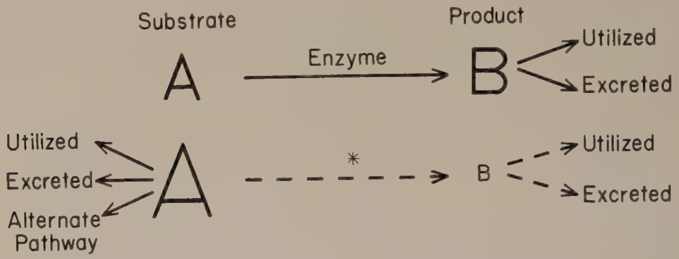

FIGURE 1. Scheme for complete and incomplete metabolism. (Reproduced with the permission of *Pediatrics*.[5])

* The enzyme or the activity of the enzyme may be lacking, resulting in a partial or complete block.

TABLE 1

HEREDITARY DISEASES IN WHICH A SPECIFIC ENZYME DEFECT HAS BEEN DEMONSTRATED

Disease	Enzyme	Tissue	Reference
Phenylpyruvic oligophrenia	Phenylalanine hydroxylase	L	Wallace *et al.*,[19] Mitoma *et al.*[20]
Alcaptonuria	Homogentisate oxidase	L, (K)	Seegmiller *et al.*,[21] La Du *et al.*[22]
Galactosemia	Galactose-1-phosphate-uridyl transferase	RBC, L	Kirkman & Kalckar[9], Kalckar *et al.*[23]
Hypophosphatasia	Alkaline phosphatase	S & WBC B, I, K, L	Rathbun[11]
Albinism	Tyrosinase (?)	Sk	Block[24]
Glycogen storage (A)	Glucose-6-phosphatase	L, K	Cori,[25] Cori & Cori[26]
Glycogen storage (B)	Brancher enzyme	L	Cori,[25] Cori & Cori[26]
Glycogen storage (C)	Debrancher enzyme	L	Cori,[25] Cori & Cori[26]
Congenital hyperbilirubinemia	Bilirubin-glucuronyl transferase	L, K	Arias & London,[27] Lathe & Walker[28]
Primaquine poisoning	Glucose-6-phosphate dehydrogenase	RBC	Carson *et al.*[29]

TABLE 2

CLINICAL FEATURES OF HYPOPHOSPHATASIA IN INFANCY

(1) Failure to thrive
(2) Slapping and flailing movements
(3) Loud high-pitched cry
(4) Deficient mineralization of bone
(5) Widening of cranial sutures
(6) Low serum alkaline phosphatase
(7) Usually hypercalcemia
(8) Usually phosphorylethanolamine in urine

cells. The disease is one with rather striking clinical characteristics, as shown in TABLE 2.

Rathbun[11] first described and named this entity in 1948 and stimulated a great deal of interest, which led to the description of about 50 cases in the years that followed. During the past 4 years 3 extensive reviews have been published.[12-14] The most important clinical manifestations are deficient mineralization of all bone in the presence of a low serum alkaline phosphatase activity. This is in contrast to rickets, a disease in which there is an elevated activity of alkaline phosphatase. McCance *et al.*[15] observed that children with hypophosphatasia have a high urinary excretion of phosphoethanolamine. This compound was detected in the urine of the child to be described and its absence has been reported in only a single instance of this disease.[13] Fraser divided his cases into 3 groups, dependent on severity, with the most severe disease and highest death rate in those with earlier onset.[13]

The diagnosis of this disease was made at The New York Hospital in a child with a history of vomiting, fever, and failure to thrive. The child was a frail, malnourished infant with widened cranial sutures and an evident rachitic rosary (FIGURE 2). Chief roentgenologic findings were poor mineralization of all bone, with marked irregularity and fading of metaphyseal margins. FIGURE 3 demonstrates these changes in the arm and leg. Initial chemistries revealed an elevated serum calcium of 6.9 and 8.3 mEq./l., a normal serum phosphorus, and an alkaline phosphatase of 0.5 to 0.8 Bodansky units. Since a deficiency in alkaline phosphatase activity in a variety of tissues has been described by several authors, in this child it appeared to be advantageous to investigate the leukocyte both histochemically and biochemically, since it is one of the more easily available cells. The histochemical examination of the white blood cells by the use of the Kaplow method,[16] utilizing the diazonium salt and naphthyl acid phosphate buffer, indicated that there was no activity in the white cells of the patient.

FIGURE 4 shows these histochemical results after 18 hours of incubation in comparison with the white cells of a normal individual that were incubated for the same period. Histochemical examination of smears obtained from the parents showed normal activity of alkaline phosphatase after 10 min. Activity of alkaline phosphatase was quantitatively determined by the method of Bessey and Lowry[17] in isolated leukocytes[18] as shown in TABLE 3. Enzymatic activity in leukocytes of the patient, in contrast to that of children of the same age, is negligible, with or without the addition of zinc or magnesium. Activity in the leukocytes of the mother is similar to that obtained from other adults. However, activity in serum obtained from the parents is lower. It may be that these findings indicate the heterozygote. In this disease the exact relationship between the deficiency in the activity of alkaline phosphatase and the clinical manifestations is not known. An estimation of the definitive association is made even more difficult by the fact that the exact function of alkaline phosphatases in the normal individual requires elucidation. It might be possible to relate the activity of alkaline phosphatase to osteoblastic activity, but this is somewhat spurious since, with cortisone administration, the child has improved markedly, clinically, and roentgenologically, while there has been no elevation of activity of alkaline phosphatase of serum, bone, or white blood cells.

Attempts in our laboratory to discern the enzymatic deficiency in white cells for other hereditary diseases such as glycogen storage disease and phenylpyruvic

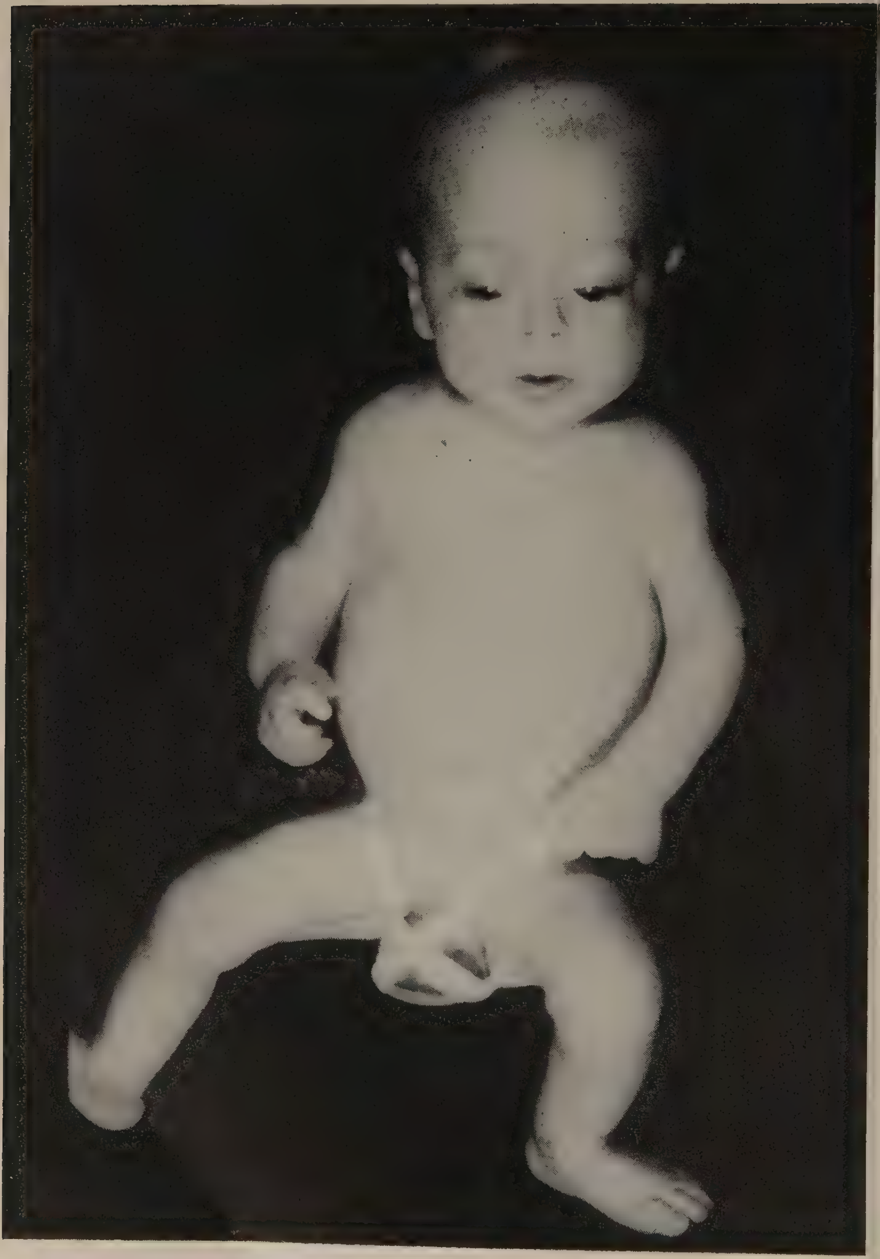

FIGURE 2. Four-month-old infant with hypophosphatasia.

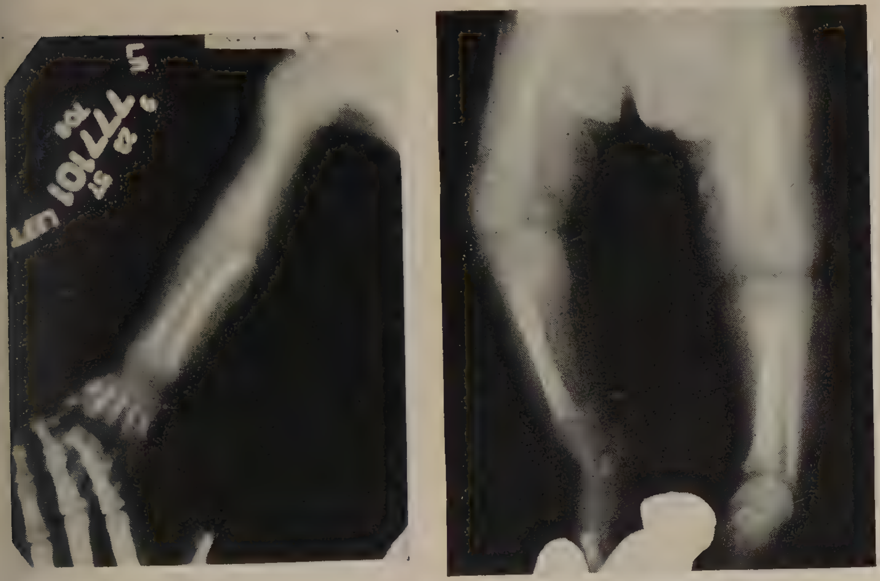

FIGURE 3. Roentgenograms of the arm and leg of an infant with hypophosphatasia

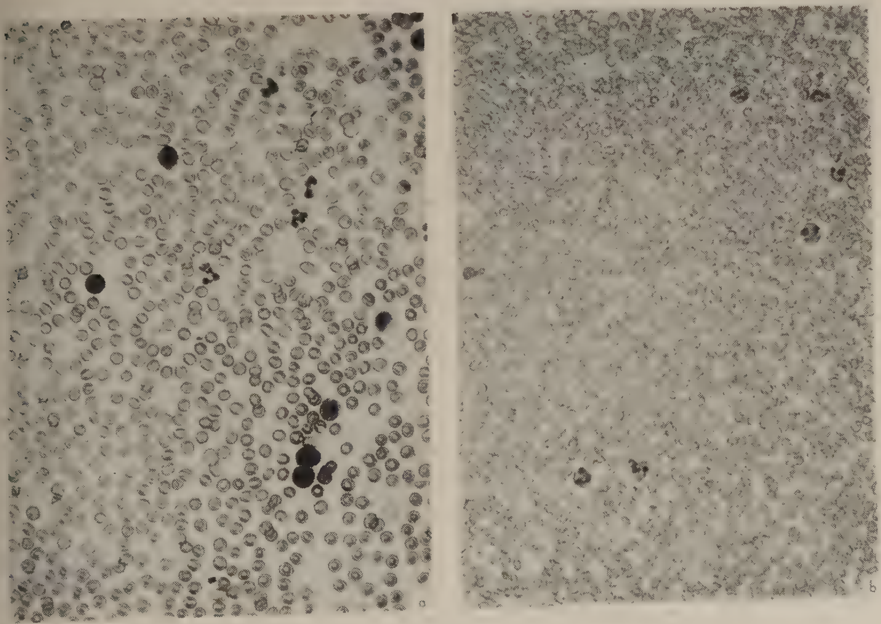

FIGURE 4. Leukocytes stained by the method of Kaplow[15] for alkaline phosphatase: *a*, normal; *b*, hypophosphatasia. ×200.

Table 3

Activity of Alkaline Phosphatase in Serum and Leukocytes from a Child with Hypophosphatasia and in Normal Individuals

	Serum (Bodansky units)	Leukocytes (μM P/hr./mg. × 10^3)		
		Citrate	+ Mg^{++}	+ Zn^{++}
Patient R.P.*	0.5–2.3	.04	.08	.08
Mother	0.8–2.2	20	56	23
Father	1.1–2.7			
Adult (normal)	1–5	24	37	24
Infant (normal)	5–15	7	15	7

* Bone from skull showed no alkaline phosphatase activity.

oligophrenia have met with failure. It is deplorable that so far only three enzymes associated with hereditary disease have been determined in blood. However, there are more instances where blood has been utilized for the determination of the accumulated metabolite, such as uric acid in gout, 17-ketosteroids in the adrenogenital syndrome, ceruloplasmin in hepatolenticular degeneration, and methemoglobin in methemoglobinemia. It is our belief that as newer and more sensitive methods become available it will then be possible to utilize blood more often and perhaps exclusively.

In summary, a case of hypophosphatasia was presented to illustrate some aspects of hereditary disease. In only a few of these diseases has the specific enzymatic defect been determined. Emphasis has been placed on the future use of blood for the diagnosis and elucidation of genetic problems with homozygotic and heterozygotic individuals.

Acknowledgment

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THE HETEROGENEITY OF LACTIC AND MALIC DEHYDROGENASE*

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In recent years biochemists and clinicians have devoted considerable attention to the enzymes lactic dehydrogenase (LDH) and malic dehydrogenase (MDH). The integrity of LDH in anaerobic glycolysis and of MDH in the tricarboxylic acid cycle is vital to cellular metabolism, and the wide distribution of these enzymes in animal tissues was therefore anticipated. Alterations in the LDH and MDH activity of serum in various disease states have been emphasized by Wróblewski and others.[1-4]

The method of starch zone electrophoresis has been used to investigate the homogeneity of LDH and MDH in human serum and red blood cells in normal subjects and in some of the conditions in which increased enzymatic activity has been reported. LDH and MDH activities in each of the segments of the starch block were determined spectrophotometrically by the methods of Wacker *et al.*[4]

The results obtained furnish evidence that LDH and MDH are electrophoretically inhomogeneous.[5, 6] Three peaks of activity for each dehydrogenase were identified in human serum and red blood cells. Each peak contained a relatively constant percentage of the total activity found in human serum. The first peak of dehydrogenase activity appeared in the beta-globulin region, the second was present in the alpha$_2$-globulin, and the third peak was found between the alpha$_1$-globulin and the albumin. The second peak of LDH activity was the largest of the three LDH peaks, whereas the first MDH peak contained most MDH activity (FIGURE 1).

Two of the three electrophoretically distinguishable proteins that reduced pyruvate also appeared to reduce oxaloacetate. The second and third peaks of LDH activity corresponded in electrophoretic mobility and *p*H optima to the second and third peaks of MDH activity, respectively (FIGURE 2). However, the first peak of LDH activity differed from the first MDH peak both in electrophoretic mobility and *p*H optimum, and therefore more than one protein must be responsible for LDH and MDH activity in this peak. It is noteworthy that the elevation of the third peak found in serum from patients with myocardial infarction and the elevation of the second peak found in the sera of patients with leukemia occur with either pyruvate or oxaloacetate as substrates (FIGURE 3).

To investigate the possibility that two of the three peaks were artifacts derived during the electrophoretic separation of serum and hemolyzate, each of the three major components of MDH and LDH activity was isolated and rerun under similar conditions. Each of the three peaks was found to have the same mobility as the starting material (FIGURE 4).

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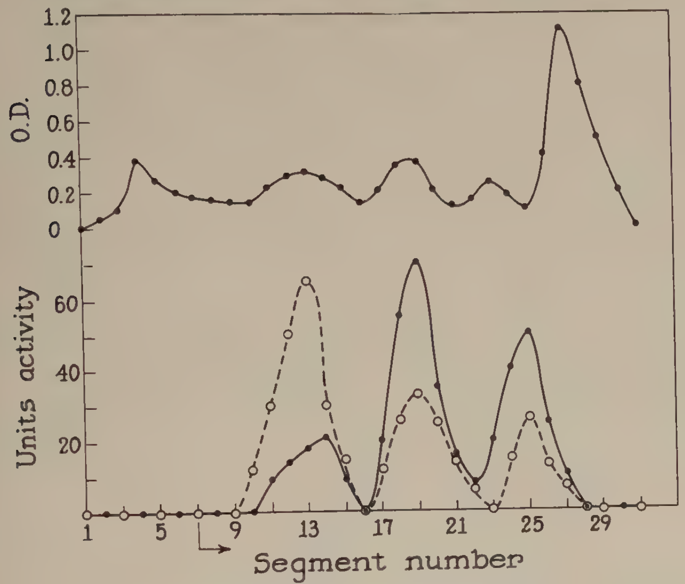

FIGURE 1. The upper curve illustrates the electrophoretic pattern of normal serum. The lower curves indicate the localization of lactic dehydrogenase (solid line) and malic dehydrogenase (interrupted line) in the serum fractions. Reproduced by permission of *The Journal of Clinical Investigation.*

Although the measurement of MDH activity in serum by the reduction of oxaloacetate is an accepted procedure,[4, 7, 8] the possible spontaneous decarboxylation of oxaloacetate to pyruvate and the simultaneous measurements of LDH and MDH activities in serum merited investigation. However, it could be demonstrated that under the conditions of these experiments spontaneous decarboxylation of oxaloacetate played a negligible role in the assay of MDH activity, since the activity with oxaloacetate as substrate LDH (rabbit muscle) was less then one twentieth that obtained with pyruvate as substrate. Furthermore, with sodium malate as substrate and DPN as coenzyme, it was found that the distribution of the three enzymes in serum was unchanged (FIGURE 5).

The presence in serum of three dehydrogenases that reduce pyruvate and of three others that reduce oxaloacetate provides another example of the heterogeneity of enzymes previously thought homogeneous. In the same year as the report of the heterogeneity of serum LDH,[5] Wieland and Pfleiderer described several electrophoretically distinct forms of LDH in each of several organs of the rat.[9] Crystalline LDH from beef heart muscle exhibits two electrophoretic peaks of activity, although monodisperse in the ultracentrifuge.[10] Chemically distinguishable forms of pepsin,[11] chymotrypsin,[12] cytochrome *c*,[13] ribonuclease,[14] lysozyme,[15] and enolase[16] have been reported.

The existence in serum of three enzymes with LDH activity and of three with MDH activity may be attributable to proteins closely related chemically and functionally or to different enzymes that react with a common substrate.

In connection with this last hypothesis, it has been reported that with the indicator dye 2,3,5-triphenyltetrazolium chloride the total serum dehydrogenas activity could be localized on filter paper to three spots approximately simila in electrophoretic mobilities to the three peaks of LDH and MDH activity.[17]

The possibility that the three peaks of LDH activity in serum may represen polymeric forms of LDH cannot be excluded,[18] although thus far sedimentatio studies of whole serum have failed to reveal more than one peak with LDH activity.[19] Isolation of the three peaks of activity and the measurement o their sedimentation rates in a density gradient would be of assistance in the clarification of this problem.

Two of the three dehydrogenases in serum and red blood cells that reduce oxaloacetate appear to be indistinguishable in electrophoretic mobility and in *p*H optima from those that reduce pyruvate. Therefore, until chemical differentiation is achieved, the possibility that the enzymes producing the second peak may be identical, and that the enzymes producing the third peak may

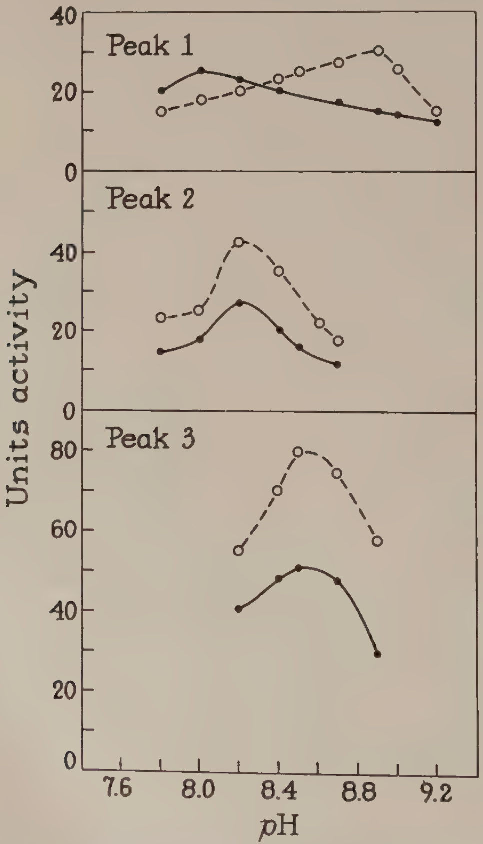

FIGURE 2. Curves illustrating *p*H optima for the three peaks of activity obtained from a red cell hemolyzate. Reproduced by permission of *The Journal of Clinical Investigation.*

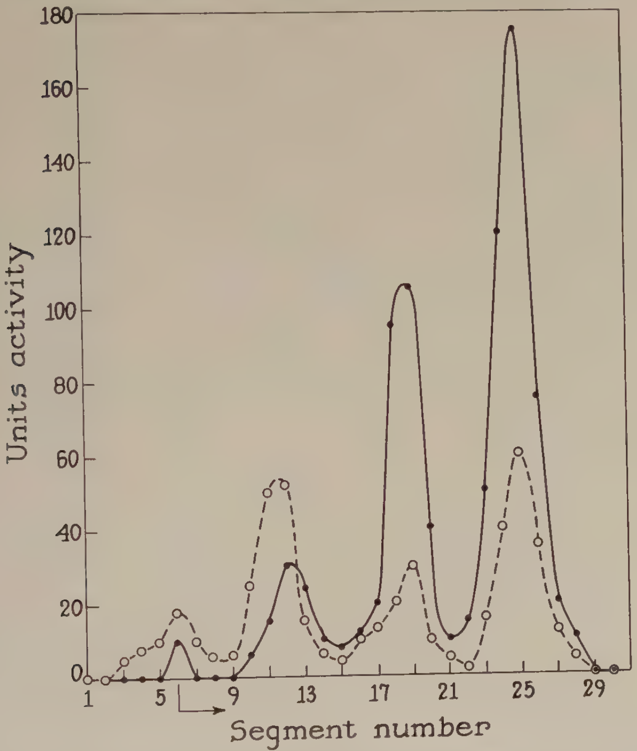

FIGURE 3. The distribution of lactic and malic dehydrogenase activity in a serum from a patient with myocardial infarction showing the selective elevation of the α_1 peak. A small quantity of activity is adsorbed at the source. Reproduced by permission of *The Journal of Clinical Investigation.*

be identical cannot be categorically dismissed. These findings are in accord with the observations that individual dehydrogenases may react with a wide range of substrates. Many different α-keto, and α, γ-diketo acids, including oxaloacetate acid, can serve as substrates for LDH obtained from rabbit muscle and beef heart.[10] Several α-hydroxy dicarboxylic acids can act as substrates for MDH obtained from pig heart.[20] Recently alcohol dehydrogenase from yeast has been shown to oxidize lactate and reduce pyruvate.[21]

The suggestion[22] that the three peaks of xanthine dehydrogenase activity, which have been demonstrated electrophoretically in rat serum, are attributable to binding by lipoproteins does not apply to LDH. High-speed centrifugation in a density gradient did not reveal significant activity in those fractions rich in lipoproteins.

Preliminary chemical and clinical characterization of the three peaks of LDH and MDH activity found in human sera and hemolyzate separated electrophoretically have been described above. Final elucidation of the nature of these three peaks remains to be achieved.

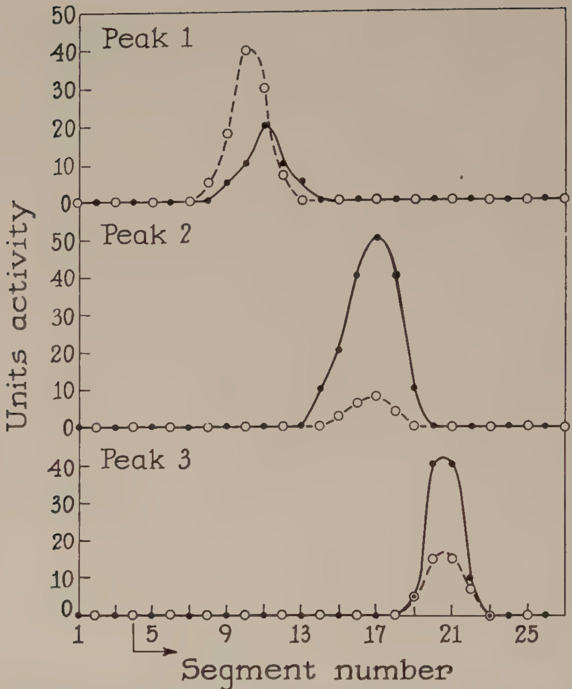

FIGURE 4. The electrophoretic pattern obtained when three peaks of activity obtained from a normal red cell hemolyzate were isolated from a starch block and rerun on a second block under identical conditions.

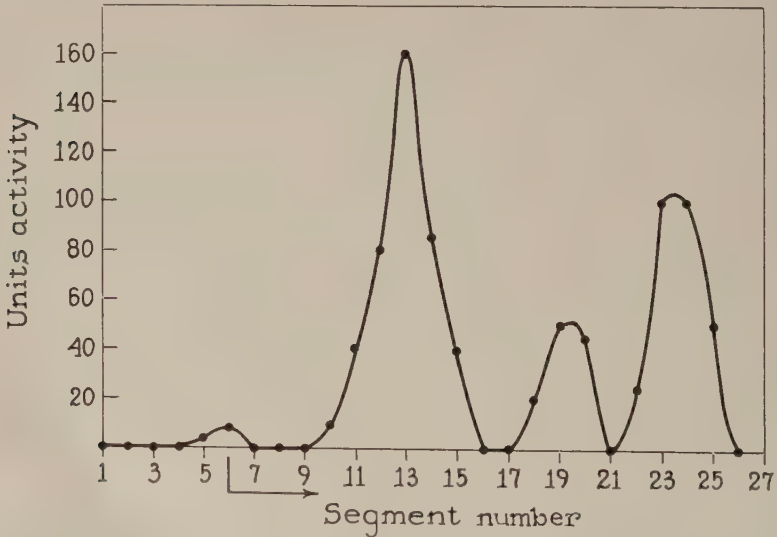

FIGURE 5. Distribution of malic dehydrogenase activity when sodium malate was used as substrate. Note the similarity of the curve with that obtained when oxaloacetate was the substrate employed (FIGURES 1 and 2). In this experiment a small quantity of activity is adsorbed at the origin. Reproduced by permission of *The Journal of Clinical Investigation.*

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DPN-DEPENDENT ENZYMES IN SERUM*

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Introduction

The significance of enzymes in cellular metabolism is well known; the specific enzyme pattern of different tissues is the basis of their physiological function. However, we know little about the enzymes in serum or their physiological importance. In recent years it has been found that normal human serum contains a number of enzymes that are part of the reaction sequences of cellular metabolic processes such as glycolysis, the citric acid cycle, and transaminase systems. The classic spectrophotometric methods developed by Warburg[1] permit us, under various physiological and/or pathological conditions, to detect the following enzymes among others in human serum:[2-6] lactic dehydrogenase (LDH), malic dehydrogenase (MDH), α-glycerophosphate dehydrogenase, glutamic dehydrogenase, isocitric dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, and pyruvate kinase. In normal serum the enzymes found are functionally inactive because of a lack of coenzymes. Under pathological conditions some enzymes can be found in pleural exudate, ascites, cerebrospinal fluid, urine, and in other body fluids.[5, 6] Under normal conditions LDH and MDH have been found to be about 3 to 10 times as high in activity as the others mentioned above. Both of these enzymes, as representatives of the diphosphopyridine nucleotide (DPN) dependent enzymes, attracted our special attention. It is the purpose of this paper to present data concerning their properties and to discuss their physiological functions.

Methods

The determination of the activity of MDH and LDH was carried out according to methods previously described.[2, 3, 6] Unless otherwise stated below, each reaction mixture contained phosphate buffer, m/15 molar, *p*H 7.4, DPNH, 4×10^{-7} mole; substrate (oxalacetate or pyruvate; 3×10^{-6} mole); 0.1 to 1.0 ml. serum (according to activity), to a final volume of 3 ml. Under these conditions the turnover of both enzymes plotted in terms of activity against serum concentration is linear (FIGURE 1). When crystalline enzyme preparations were added they could be recovered in respective proportions. No differences in activity are apparent when the phosphate buffer is replaced by glycyl-glycine buffer or triethanolamine buffer. Hemolytic sera were not included in the investigation. For studies of enzyme properties the sera were dialyzed 12 hours with isotonic saline solution at 6° C. The determination of the activity of the other above-mentioned enzymes was carried out according to the method of Warburg[1] or that of Colowick *et al.*[7]

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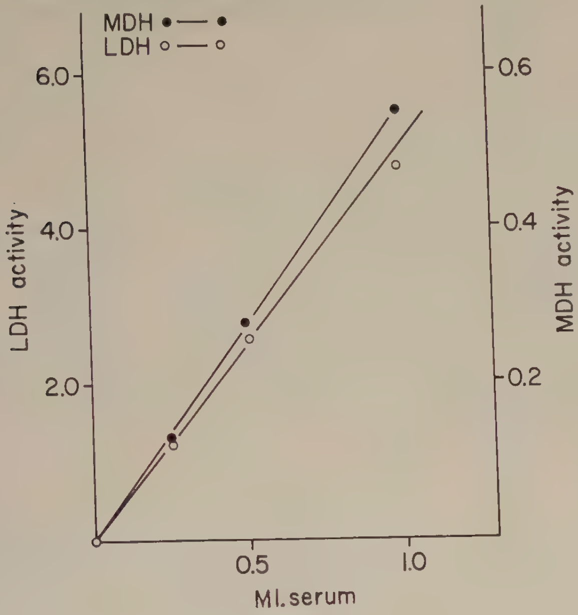

FIGURE 1

The optical determinations were carried out with an Eppendorf photometer at 366 mμ or a Beckman spectrophotometer DK 4 at 340 mμ, 22° C. The *p*H activity curves were drawn from measurements obtained when a phosphate buffer or a glycyl-glycine buffer was used. In these preparations sodium pyruvate;* also enzymatic hydrated DPN (95 per cent preparation No. 15142); oxalacetate, LDH (preparation No. 15145); and MDH (preparation No. 15048) was used.† The paper electrophoresis of LDH was carried out in an electrophoresis apparatus‡ running 6 to 12 hours at 110 v., set at 25° C., in Veronal buffer (0.033 molar, *p*H 8.6). Next, 0.1 ml. serum was pipetted on paper strips of 12-cm. width. After electrophoretic separation a small strip 0.5 cm. in width was cut out for identification of the serum protein fractions by treatment with Amidoschwarz (E. Merck, Darmstadt, Germany, Preparation No. 1167). The other part of the paper strip was cut into small pieces corresponding to the albumin, α_1-globulin, α_2- and β-globulin, and γ-globulin fractions. Each part was eluted with 0.5 molar ammonium sulfate solution and checked for enzyme activity in the usual way. Because of low activity the test was usually carried out at 37° C. for 30 to 60 min. From 60 to 80 per cent of LDH activity could be recovered. Most of the MDH activity was lost during this procedure.

In all optical tests the reaction time required for a change in optical density

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* Produced by E. Merck, Darmstadt, Germany.

† Donated by C. F. Boehringer & Soehme, Mannheim, Germany.

‡ The Elphor, manufactured by Bender & Hobein, Munich, Germany.

(O.D.) of 0.1 was determined. An activity unit (U) is defined according to Beisenherz *et al.*[8] as follows:

$$U = \frac{100}{\text{time in seconds, required for change in O.D. of 0.1 to take place}}$$

At 366 mμ (distance = 1 cm.) this unit is equal to a turnover of 1.76×10^{-5} mole/min. The calculation of the Michaelis constant was carried out according to Lineweaver and Burk.[9]

According to the work of Vallee and Wacker[10] heavy metals exert an influence on LDH activity. In agreement with their results we found a decrease of 48 per cent in serum LDH activity on addition of a chelating agent in the form of neutral Versene (0.04 molar). No such influence on serum MDH activity has yet been perceived. Since methods of controlling these factors in the heterogenous serum were not practicable, none were incorporated in this investigation.

Results

Enzyme Activity under normal conditions. Under normal conditions 5.95 (S.D. ± 1.5, n = 15) units of LDH and 1.5 (S.D. ± 0.5, n = 14) units of MDH were found in serum (TABLE 1). In this table activities of other DPN-dependent enzymes are also shown. Their properties were not studied in more detail. Further work is in progress. Measures of the concentration of enzyme protein of LDH and MDH on the basis of known turnover numbers (LDH calculated according to Kubowitz and Ott[11]) and MDH calculated according to Ochoa[12] give a value of 310 γ protein per liter of serum for LDH and 78 γ protein per liter of serum for MDH. Undoubtedly, this type of calculation embodies a high degree of uncertainty. Furthermore, it should be noted that a considerable concentration gradient exists between the intracellular space and the serum. The concentration gradient of LDH between the serum (activity per liter) and erythrocytes (activity per liter intracellular fluid) was found to be 260. Similar ratios hold good for other enzymes. Thus, in the case of aldolase, we found a gradient of 108. Warburg and Christian[13] and Bodansky[14] give similar results for other enzymes and tissues.

TABLE 1

DPN-DEPENDENT ENZYMES IN SERUM

Enzyme	Concentration under normal conditions* units/ml.
Lactic dehydrogenase	5.95 ± 1.5
Malic acid dehydrogenase	1.5 ± 0.8
1-Glutamic dehydrogenase	>1.0
α-Glycerophosphate dehydrogenase	>1.0
Glyceraldehyde-3-phosphate dehydrogenase	>1.0

* Undialyzed, without Versene.

Enzyme Activity under pathological conditions. Different authors have found that activity rates vary in pathological conditions. In addition to the well-known relationships in the case of myocardial infarction,[15] liver disorders,[2] myopathia,[16] and certain malignancies,[2, 17, 18] another was established in the case of pernicious anemia.[2] As shown in FIGURE 2, the red blood count varies inversely with LDH activity. It should be noted that a 50 per cent decrease in the erythrocyte count is proportional to a fourfold increase in activity. A similar relationship has been found between MDH activity and the basal metabolism rate (B.M.R.) in the case of thyrotoxicosis.[6] Furthermore, our interest was attracted by the finding of enzyme activity in extracellular fluids other than the serum.[3-6] Both enzymes appear among others in exudates of the pleural, pericardial, or abdominal cavity, and are also found in cerebrospinal fluid and urine when the respective tissues develop pathological conditions. At this time I shall not discuss the significance of these results in respect to the problem of malignancy.

Properties and differentiation of enzymes. The extraordinary constancy of enzyme activity under normal conditions as well as the variation of enzyme activity in pathological disequilibria is remarkable. These phenomena raise problems concerning the sources, function, and regulation of enzymes in serum. LDH and MDH activities are found in all types of body cells. There they exist as parts of the specific enzyme pattern in accordance with the specific physiological function of each tissue and organ. Therefore, the problem of the differentiation of serum enzymes with regard to their origin might be in-

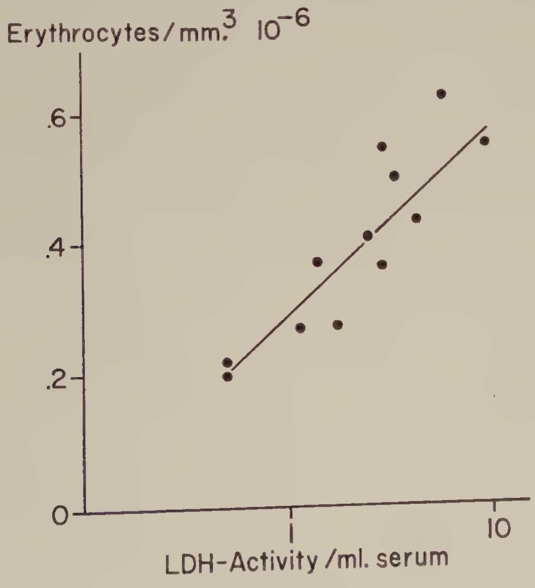

FIGURE 2

vestigated by applying the classic concept of specificity to organ enzymes. How far these enzymes can be differentiated on the basis of functional or structural properties is a matter that requires investigation. For this reason we studied the following physicochemical and biochemical properties of the enzymes under normal and pathological conditions: *p*H optima, substrate affinity, electrophoretic behavior, and inhibition by sulfite. While our study was under way Wieland and Pfleiderer[19] were able to demonstrate that samples of crystalline LDH obtained from various organs and different animal sources could be differentiated, using these same characteristics as criteria.[20]

pH Optima. In a previous publication I mentioned the *p*H optima of LDH in mixed sera from different sources as being 9.0.[2] In later studies, however, I realized that the *p*H optima differ under various conditions. In FIGURE 3 the *p*H optima of serum LDH for the reaction of pyruvate hydration are given in two pathological states. The optimum in the cancerous condition (Tu) is shifted to the left; in cases of pernicious anemia (Pa) it is shifted to the alkaline side. Additional values are given in TABLE 2. In this case the difference in activity is also expressed as a quotient formed by the units of activity at *p*H optimum over the units at *p*H 7.5. Tentatively, one might describe at least 3

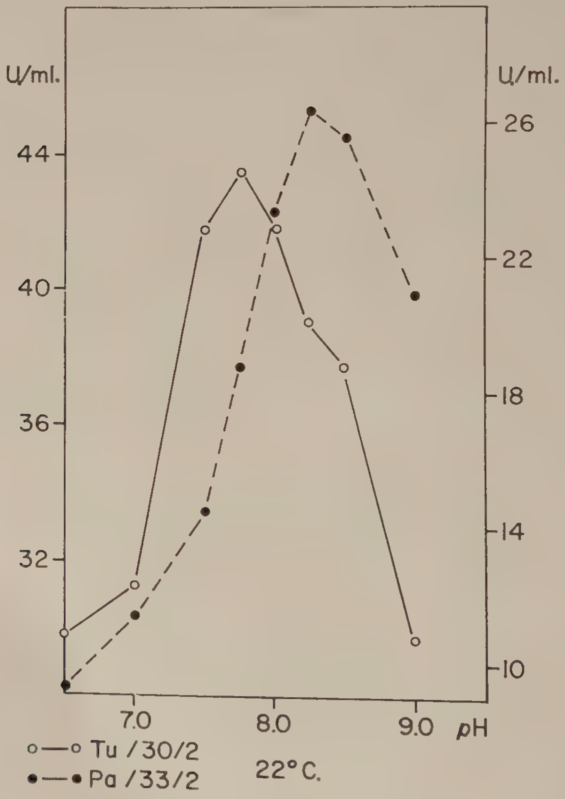

FIGURE 3

TABLE 2

LDH OPTIMA UNDER DIFFERENT CONDITIONS

No.	Type	U/ml. serum	pH optim.	Q_{pH}	Remarks
19/2	Normal	6.6	8.0	1.03	Undialyzed
21/2	Tumor	118.0	8.0	1.18	Arterial
20/2	Tumor	156.0	7.5	1.0	Undialyzed
30/2	Tumor	43.5	7.75	1.0	
22/2	Myocardial infarction	13.0	8.0	1.2	Undialyzed
20/2	Myocardial infarction	15.0	8.5	1.6	
37/2	Myocardial infarction	26.4	8.25	1.8	
33/2	Pernicious anemia	54.0	8.5	1.84	
32/2	Hemolytic serum	250.0	8.5	1.4	
31/2	Hemolytic serum	23.7	8.38	1.4	Undialyzed
G103a	Pernicious anemia	16.0	9.0	7.3	Undialyzed + sulfite

$$Q_{pH} = \frac{\text{U at } p\text{H} - \text{optimum}}{\text{U at } p\text{H } 7.5}$$

optima of different types. Certainly, the measure of a pH optimum is to be taken as a relative and not an absolute value of the heterogenous serum. I make the assumption that a mixture of various enzymes is present, and that under pathological conditions one or the other is increased in concentration. Speculation leads to the hypothesis that the more alkaline type might be a consequence of hemolysis, with an increase in LDH from erythrocytes as its source. The more acidic type might be considered as derivative of skeletal muscle enzyme.

Substrate affinity. Another aspect of our studies concerned the substrate affinity. A comparison between the Michaelis constant (K_m) of serum LDH for pyruvate in different clinical conditions revealed significant differences, as shown in TABLE 3. It is interesting to note that different types, with higher and with lower affinity, again become evident.

TABLE 3

SUBSTRATE AFFINITY FOR PYRUVATE OF SERUM LDH*

No.	Type	K_m mol./l.
41/2/1	Mixed serum	2.86×10^{-4} 1.3×10^{-4}
41/2/2	Hemolytic serum	2.5×10^{-4} 2.25×10^{-4}
41/2/3	Tumor	2.0×10^{-4} 2.2×10^{-4} 2.35×10^{-4}
41/2/4	Pernicious anemia	6.67×10^{-4} 6.7×10^{-4}
41/2/5	Myocardial infarction	9.1×10^{-5} 8.35×10^{-5}

* Computed according to Lineweaver and Burk.[9]

Table 4
Electrophoretic Mobility of Serum LDH

No.	Type	U/ml.	Per cent activity in					Remarks
			γ-Glob.	β-Glob.	α_2-Glob.	α_1-Glob.	Albumin fraction	
1	Tumor	52		26	43	22	9	Bronchial
2	Tumor	60	17	22	21	20	20	Bronchial
3	Tumor	50	31	16	27	15	11	Bone marrow
4	Pernicious anemia	54	8	7	21	45	20	
5	Myocardial infarction	31		6	10	50	35	
		26		24	38	38		

Electrophoretic mobility. Of further interest was the localization of LDH activity in the electrophoretic serum fractions. In this study I made use of paper electrophoresis. The usefulness of this method is limited by the small quantities of serum sample that are processed and the loss of enzyme activity through inactivation by the paper. Therefore, in normal serum a clear localization of enzyme activity cannot be established with this method. Under pathological conditions with high enzyme activity, however, distinct peaks of LDH activity can be read, as seen in TABLE 4. This finding agrees in principle with those recently published by Vesell and Bearn[21] and Sayre and Hill.[22] Here, again, several different fractions of serum LDH become apparent. The separation of serum MDH is not so clear-cut, especially because of a very poor recovery of enzyme activity, but it also shows the same trend.

Sulfite inhibition. In older studies LDH and MDH activity measurements were carried out with DPNH that was obtained by chemical reduction of DPN through sodium hydrosulfite according to Warburg.[1] Later it became evident that enzymatically reduced DPN yields higher measures of activity. This leads to the realization that sulfite impurities of this DPNH constitute a strong inhibitor of both enzymes. This phenomenon was recently described in detail by Pfleiderer *et al.*,[23] who demonstrated that sulfite inhibition is brought about by a sulfite-DPN adduction that competes for the site of substrate at the enzyme surface. Studying this inhibitor in more detail, we found several degrees of inhibition of serum LDH and serum MDH that varied with the respective conditions as shown in TABLES 5 and 6. This again leads us to the conclusion that we are dealing with different types of the same enzyme. With their crystalline preparation Wieland and Pfleiderer[19] were able to show that the degree of sulfite inhibition bears a relationship to the electrophoretic mobility of their enzymes, the fast-moving enzymes showing the strongest inhibition on the basis of their greater negative charge. I conclude, therefore, that the varying inhibition of serum enzymes reflects their varying electrophoretic mobility. If the conclusion is correct, the degree of inhibition must be proportional to the enzyme activity of a homogeneous fast-moving protein. This

TABLE 5
SULFITE INHIBITION OF SERUM LDH

No.	Type	U/ml.	Per cent inhibition
1	Tumor	156.0	24.0
2		62.0	38.0
3		52.0	41.0
4		44.0	13.0
5		44.0	13.0
6		32.0	9.0
7		16.0	41.0
8		10.0	7.0
1	Pernicious anemia	57.0	25.0
2		15.0	7.0
1	Infarction	112.0	43.0
2		31.0	20.0
3			
4		19.0	31.0
5		11.7	11.7

TABLE 6
SULFITE INHIBITIONS OF SERUM MDH

No.	Type	U/ml.	Per cent inhibition
1	Tumor	149	25
2		44	13
3		35	17
4		24	20
5			
6			
1	Pernicious anemia	57	38
2		18	42
1	Infarction	11	
2		13	10
3		17	14
4		21	14
5		24	34
6		29	21
7		36	38
8		52	59

condition might hold for the fast-moving LDH component in the case of myocardial infarction. This is demonstrated in FIGURE 4, where units of activity in sera of this clinical condition are plotted against the percentage of sulfite inhibition. This relationship can also be seen by comparison of the percentage of inhibition with that of the fast-moving component of total activity, as shown in TABLE 7.

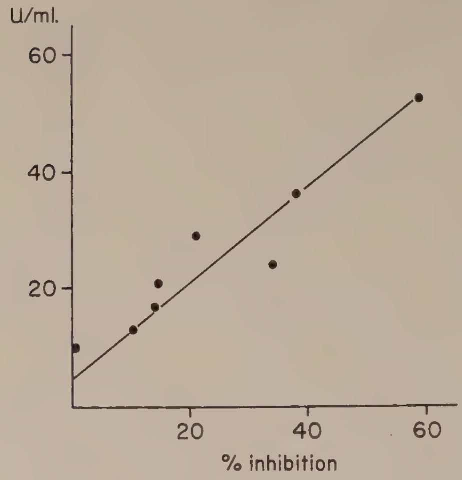

FIGURE 4

TABLE 7
SULFITE INHIBITION AND LDH ACTIVITY IN ALBUMIN FRACTION

Type	U/ml.	LDH activity in albumin fraction in per cent of total act.	Per cent inhibition
Tumor	60	20	24
	52	9	13
	50	11	9
Pernicious anemia	54	20	25
Infarction	31	35	20
	26		

Discussion

Blood serum provides an interesting material for studies on cellular turnover, enzyme turnover, enzyme specificity, and their use as diagnostic indicators. The constancy of enzyme activity under normal conditions reflects a mechanism that releases the enzyme from some source at a constant rate. I have disregarded the possibility that degradation or excretion plays a significant role in the maintenance of enzyme concentration (see also Warburg and Christian).[13] According to discussion of the matter by various authors,[2-6, 13, 24-26] there are two mechanisms by which enzymes can be released from tissues: (1) degradation and lysis of cells, with a loss of cellular enzymes as a phenomenon of either physiological cellular turnover or of pathological conditions (such as anaerobiosis and membrane damage); and (2) secretion of enzymes by cells in a physiological manner or as a pathological process.

The physiological disintegration of erythrocytes and other blood cells that leads to their normal end state might play a role in the maintenance of normal enzyme concentration, as may also that of the intestinal mucosa, whose high cellular turnover is indicated by its high mitotic rate. While there is insufficient evidence to support either of these possibilities over the other and the relatively higher concentration of LDH in the portal system speaks for both,[27] I tend toward the thesis that it is the degradation of erythrocytes that supplies the major part of normal serum enzyme. The cellular turnover rate of muscle and nervous tissue is minimal and might be neglected; in addition, we have as yet been unable to determine any arteriovenous differences in enzyme activity either in normal persons or in tumor patients, which indicates the participation of both tissues.

The possible physiological internal secretion of enzymes is a highly interesting aspect of the matter, but evidence for this is lacking at present. Such processes would include a mechanism for the uptake of enzymes by cells, which was actually observed by Warburg *et al.*[28] in the case of ascites tumor cells.

Under pathological conditions serum enzymes come from different sources, depending upon where the pathological state develops. In the case of pernicious anemia a major source may again be the red blood cells and their precursors. It is known that in this condition the erythrocyte degradation rate is accelerated (see Heilmeyer & Begemann[29]). Increased erythrocyte degradation is followed by increase in serum LDH activity; FIGURE 2 clearly indicates this relationship. However, a 50 per cent decrease in the red cell count yields a far greater (fourfold) increase in activity than would at first be expected. This result seems to indicate that not only is the red cell system involved, but that other cells also add to the concentration of serum enzyme (Singer *et al.*[30]). Another explanation might be found in the assumption that the enzyme content of the individual erythrocyte rises in proportion to its increased degradation rate in this condition. In the case of the correlation between the B.M.R. in thyrotoxicosis and MDH activity the mechanism of enzyme release may be found in the high intracellular protein turnover and also in damage suffered by membranes, both of which occur as the result of the action of the thyroid hormone on cellular oxidation.

Under normal and pathological conditions the different DPN-dependent enzymes in serum should reflect the material from which they come in their concentrations. A clear identification of the source of an enzyme on the basis of a comparison of the ratio of its concentration to those of other serum enzymes with the ratio of its respective tissue enzymes has so far not been satisfactory. Furthermore, each calculation of serum enzyme concentrations on the basis of the concentration in tissues is difficult because of "impurities," which consist in the fact that, especially under pathological conditions, the capillaries are loaded with blood cells rich in many of the enzymes involved. Our knowledge of the intracellular localization of MDH might also be revised. Delbrück and Bücher[31] found that in heart muscle almost 100 per cent of this enzyme is dissolved in the cytoplasm. In addition, I found that MDH activity in erythrocytes was at least as high as LDH activity. Obviously, therefore, MDH is to be considered a cytoplasmatic and not a mitochondrial enzyme, at least in some cases.

An identification of serum enzymes may be possible on the basis of their heterogeneity. It is highly interesting that four different methods reveal differences in physical and biochemical properties, but not in reaction mechanisms. These findings are in accord with similar experiments recently described by Wieland and Pfleiderer,[19] by Pfleiderer and Jeckel,[20] by Hill,[32] and also with earlier observations made by Kaplan *et al.*[33] These findings suggest that the concept of enzyme specificity goes far beyond the substrate specificity and is to be extended to cover physiological specificity in the broadest sense. Given further investigation along these lines, with the inclusion of other additional enzymes I believe that the use of such properties, including immunological features (for the differentiation of these enzymes and the identification of their source in pathological states) is practicable as a diagnostic indicator. Of course, much more clinical experience in this direction must be collected.

More recently we have been able to demonstrate that LDH from different human tissues can be distinguished on the basis of their heterogeneity in the different properties given above.

Summary

(1) Normal values for serum LDH and serum MDH were established.

(2) Pathological values are given, especially with respect to serum LDH in pernicious anemia.

(3) As an approach for differentiation of serum enzymes, the properties of LDH and MDH were studied. It was found that *p*H optima, substrate affinity (for pyruvate), electrophoretic behavior, and sulfite inhibition of serum LDH differ under various pathological states.

(4) The significance of these findings is discussed.

Acknowledgments

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FURTHER STUDIES OF THE FRACTIONATION OF LACTIC DEHYDROGENASE OF BLOOD

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Hill and Levi[1] first showed that lactic dehydrogenase (LDH) activity of human blood serum is elevated in many individuals with neoplastic disease. Various properties of the enzyme have been described[2, 3] and correlations have been made of serum LDH activity with the clinical status of a number of leukemic patients.[4, 5] Hess and Gehm[6] reported an increase of serum LDH in patients with pernicious anemia, and Wróblewski and LaDue[7] found an elevation after myocardial infarction and in several other pathological conditions. Reports of a number of related clinical investigations followed.

Serum LDH activity was found to be correlated directly with neoplastic growth and regression in mice.[8, 9] In mice with leukemia there was a profound elevation in serum LDH prior to the appearance of any other detectable indications of the disease.[10-12] The level of serum LDH activity proved to be a sensitive indicator in following the course of the L4946 transplantable lymphatic leukemia in AKR mice.[10, 11]

It was found that serum lactic dehydrogenase could be separated into a number of components[13, 14] by paper electrophoresis and gradient elution procedures and that several factors could influence the number of components observed.[14] The present study is a report of an investigation of some of the interrelationships of the various fractions prepared by continuous-flow paper electrophoresis.

Methods and Materials

Electrophoretic separation of LDH components was carried out at room temperature in the Spinco continuous-flow paper electrophoresis unit, Model CP, as previously described.[14] Prior to fractionation serum was diluted 1:10 (for high activity serum) and 1:5 (for low activity serum) with barbital buffer, *p*H 8.6, ionic strength 0.02. The diluted serum was dialyzed against the same buffer overnight at 4° C. LDH activity was determined by a spectrophotometric method as described previously.[1]

Erythrocytes were prepared from heparinized blood. The cells were washed twice with a volume of isotonic saline equal to the original volume of plasma. Four volumes of water were added to the washed erythrocytes; the mixture was then frozen and thawed three times and centrifuged. The supernatant solution was diluted 1:25 with barbital buffer and dialyzed. Leukocytes were treated in the same manner except that, after thawing, the mixture was homogenized in a ground glass tissue grinder. Platelets were not washed. Otherwise, they were treated in the same manner as leukocytes. Ascitic

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fluid was centrifuged immediately after withdrawal from C57 black mice bearing the Ehrlich ascites tumor in order to minimize leakage of LDH activity from the cells.[3] The cells were washed twice with isotonic saline and centrifuged immediately. The washed cells were prepared for electrophoresis in the manner described for leukocytes and the ascitic fluid was diluted as previously described for high activity serum.

Michaelis-Menten constants (K_m) for pyruvate were determined as a function of *p*H employing a suitable concentration range of pyruvate for each value of *p*H. The reaction was initiated by the addition of reduced diphosphopyridine nucleotide (DPNH) in 0.1M phosphate buffer to give an initial concentration of 1.53×10^{-4}M, allowed to proceed for 10 min. at 37° C., and the activity estimated spectrophotometrically by the decrease in absorption at 340 mμ.[1] In all determinations the LDH activity was zero order and proportional to the amount of enzyme employed.

Studies of heat inactivation were carried out with fractions II, III, and IV, diluted with barbital buffer, *p*H 8.6, ionic strength 0.02, so that each fraction had approximately the same enzymatic activity. Samples were heated for 5 min. at various temperatures in a constant temperature bath, cooled quickly, and assayed for residual LDH activity in a reaction mixture containing 1.53×10^{-4}M DPNH and 8.1×10^{-4}M pyruvate in 0.1M phosphate buffer, *p*H 7.8. The reaction was allowed to proceed for 30 min. at 37° C. In some instances reduced glutathione (GSH) was added to a final concentration of 5×10^{-3}M before heating.

Results

A typical electrophoretic pattern obtained with high activity serum from a patient with granulocytic leukemia is shown in FIGURE 1. Tubes containing enzymatic activity were pooled and designated as fractions I, II, III, and IV. Separated active fractions, when recombined and subjected to electrophoresis under the same conditions, gave patterns identical with those obtained in the initial run. When any individual fraction was added to low-activity whole serum an elevation was observed only in the fraction corresponding to the added material.

The relative proportions of the total activity found in the individual fractions obtained from various samples of serum are shown in TABLE 1. Individual and pooled samples of normal serum gave similar patterns, which were reproducible upon replicate fractionations of the same sera.

Fraction I, present in all sera, accounted for only a small percentage of the total activity. Fractions III and IV from normal serum each accounted for approximately 40 per cent of the total activity. Fractionations of individual and pooled sera from children with lymphocytic leukemia gave reproducible patterns in which fraction III contained more than 50 per cent of the total activity, and fraction IV contained slightly less than one half the activity of fraction III. The total LDH activity of the sera from children used for the above fractionations was 50 to 100 times the activity of that of serum obtained from normal children of the same age. The patterns of sera of patients with

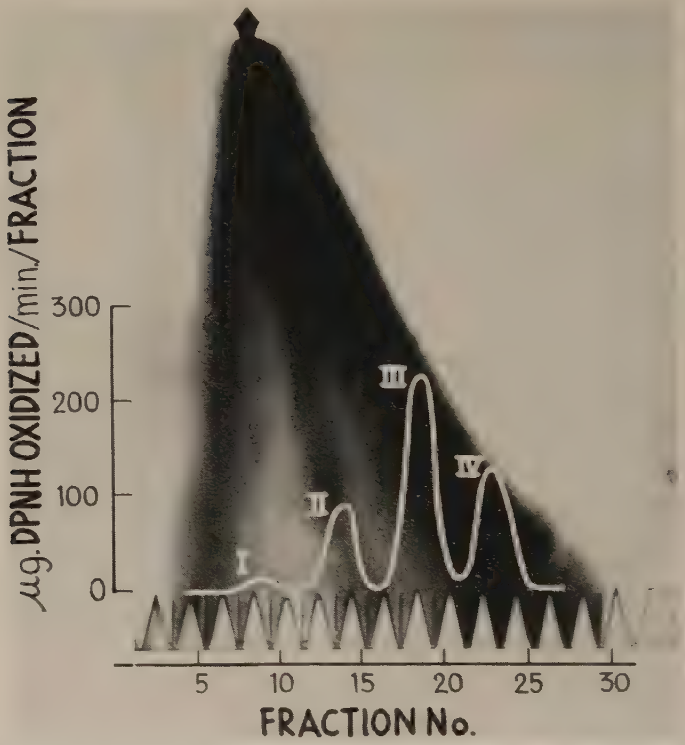

FIGURE 1. Electrophoretic separation of serum from a patient with granulocytic leukemia. The stained areas show the protein components; the superimposed curves correspond to the levels of LDH activity.

leukemia shown in TABLE 1 are typical of those obtained in 20 fractionations. The serum LDH from an adult patient with granulocytic leukemia undergoing treatment with dimethyl busulfan (dimethyl Myleran) was in the normal range for total LDH activity. However, the distribution of electrophoretically separated components resembled those obtained with sera of children with lymphocytic leukemia rather than the normal adult pattern. The serum of a patient with cancer of the testes with widespread metastases (total LDH activity approximately 100 times normal) and a patient with Tay-Sachs disease (LDH activity three times normal) showed electrophoretic patterns that differed from those obtained with normal or leukemic sera.

The relative activities of the various fractions obtained from the formed elements of blood are compared with those from normal serum in TABLE 2. Fraction I was observed only in platelets. Greater proportions of fraction II were observed consistently in all of the formed elements than in serum. This fraction accounted for over 50 per cent of the total LDH activity in leukocytes. Leukocytes from a patient with chronic lymphatic leukemia showed an electrophoretic pattern similar to that of leukocytes from a healthy individual.

TABLE 1

LDH ACTIVITY IN SERUM FRACTIONS

Serum	Percentage of total activity			
	I	II	III	IV
Normal*	0.1	20.8	38.5	40.6
Lymphocytic leukemia†	1.2	16.0	56.2	27.8
Granulocytic leukemia (Rx)‡	0.5	15.6	58.7	25.2
Cancer testes (Rx)§	0.3	5.4	41.2	53.1
Tay-Sachs disease	1.2	10.3	48.0	40.5

* Serum from apparently healthy adult male individuals.

† Typical pattern from sera of children with lymphocytic leukemia. Serum LDH 50 to 100 times normal.

‡ Serum from adult male with granulocytic leukemia obtained during course of treatment with Myleran at which time the patient was in remission.

§ Male, 44 years of age, with cancer of the testes with widespread metastases to liver, abdomen, and lungs. Liver damage. Serum LDH approximately 100 times normal.

TABLE 2

LDH ACTIVITY IN FORMED ELEMENTS OF BLOOD COMPARED TO NORMAL SERUM

	Percentage of total activity			
	I	II	III	IV
Serum (normal)	0.1	20.8	38.5	40.6
Erythrocytes*	0	39.8	31.8	28.4
Platelets**	2.3	36.8	44.6	16.3
Leukocytes (N)†	0	62.6	26.7	10.7
Leukocytes (gran. leuk.) (Rx)‡	0	100.0	0	0
Leukocytes (lymph. leuk.) (Rx)§	0	58.0	30.5	11.5

* From apparently healthy adult individual.

** From apparently healthy individual. Less than 1 per cent leukocytes.

† From an apparently healthy individual. Approximately 100% polymorphonuclear leukocytes.

‡ From a 42-year-old male with chronic granulocytic leukemia. Under treatment with dimethyl Myleran.

§ From 77-year-old male with chronic lymphocytic leukemia. Heavy corticosteroid treatment. Serum LDH 4 times normal. Leukocyte count approximately 500,000.

Leukocytes from a patient with chronic granulocytic leukemia gave an electrophoretic pattern in which fraction II accounted for the total activity found.

The electrophoretic pattern obtained from the sera of normal C57 black mice was compared with that obtained from the fluid and cells of Ehrlich ascites tumor grown in the same strain of mice (FIGURE 2). Only a single fraction was found for the LDH in ascitic fluid and tumor cells despite the fact that both contained high levels of activity. Electrophoretic mobilities of the three fractions obtained from mouse serum were roughly comparable to those of fractions II, III, and IV of human serum. The results show the major portion of the activity was in fraction II, with relatively minor contributions of III and IV. The single fraction obtained from ascitic fluid and cells corre-

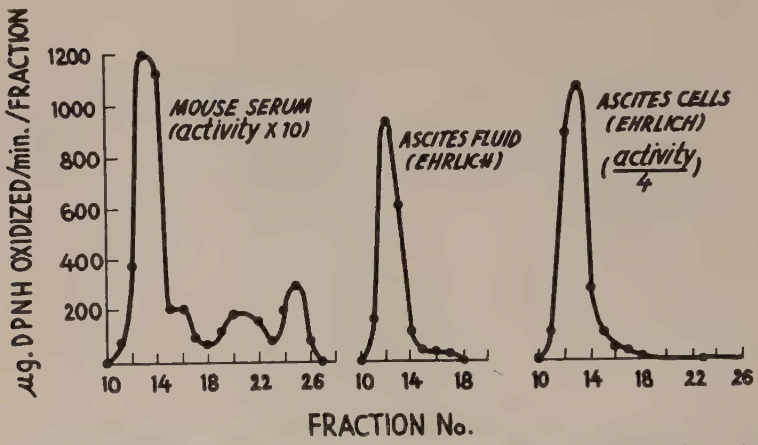

FIGURE 2. Electrophoretic fractionation of LDH in mouse serum, ascitic fluid, and ascites cells (Ehrlich tumor).

TABLE 3
MICHAELIS-MENTEN CONSTANTS (K_M) FOR PYRUVATE

	K_m (pyruvate) × 10^4 M/liter		
	*p*H 7.1	*p*H 7.6	*p*H 8.0
Serum (normal)	0.6	1.5	5.5
Serum (leukemic)	1.0	1.8	5.8
Fraction II	0.9	2.5	5.2
Fraction III	0.6	1.8	—
Fraction IV	0.5	2.1	4.9

sponded approximately in mobility to a single component observed in leukocytes in the patient with granulocytic leukemia (see TABLE 2).

In an attempt to characterize fractions II, III, and IV obtained from human serum studies have been made of K_m for pyruvate. It was found that the K_m values are dependent on *p*H, increasing values (lower affinity of substrate for enzyme) being observed at higher *p*H values in the range of 7.0 to 8.0 (TABLE 3). The preliminary results indicate that these three fractions cannot be distinguished by these measurements.

The results in FIGURE 3 show that glutathione greatly enhanced the heat stability of fractions II, III, and IV, suggesting that heat inactivation of the LDH activity of serum is related to the sulfhydryl nature of the enzyme. The greater heat stability of fraction IV is possibly attributable to the presence in this fraction of large quantities of albumin which appear in the tubes containing this fraction.

Discussion

The electrophoretic fractionation of serum LDH into several active fractions[13, 14] and the elevation of specific components in leukemia and myocardial

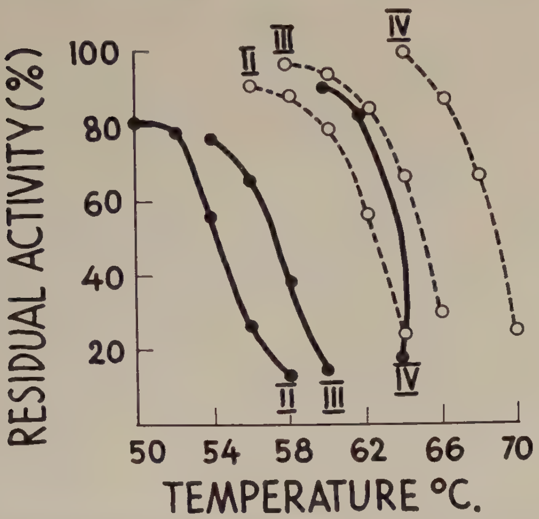

FIGURE 3. Heat inactivation of serum fractions. The solid lines describe the loss of activity of fractions II to IV as isolated by electrophoresis; the dotted lines, duplicate samples heated in presence of 5×10^{-3} M GSH.

infarction[13] warrants a more critical investigation of the serum enzyme. Hill *et al.*[15] reported that LDH of serum and lysed erythrocytes behaved as a monodisperse compound in sedimentation studies. Vesell and Bearn[13] were able to separate the LDH of serum and lysed erythrocytes into three active components by electrophoretic fractionation, and suggested that these fractions might correspond to different forms of LDH elaborated by different tissues. Sayre and Hill[14] also found several active fractions in electrophoretic and constant gradient ammonium sulfate elution studies, and suggested that serum LDH might consist of a single molecular species that might exist in different states of aggregation.

At the present time no conclusions can be drawn regarding the relationship between the electrophoretically detectable fractions in serum and those of the formed elements of blood. Similar studies will be made of LDH of various tissues.

Summary

A simple, reproducible method for the electrophoretic fractionation of serum lactic dehydrogenase is described. Electrophoretic patterns are shown for sera and formed elements of blood of normal and leukemic humans, and the serum and fluid and cells of Ehrlich ascites tumor of C57 black mice.

The values of K_m (pyruvate) for whole human serum and fractions II, III, and IV were similar and increased with increasing *p*H between *p*H 7.0 and 8.0. The heat stability of these three fractions was studied and found to be increased by the addition of reduced glutathione.

Acknowledgment

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THE MODE OF ACTION OF HEART MUSCLE LACTIC DEHYDROGENASE*

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The work to be outlined in this report has been in progress for several years. While a great deal is now known of the mode of action of the simple diphosphopyridine (DPN)-dependent dehydrogenases, very little was known of these systems at the outset of this investigation. Our early work was greatly facilitated by the publications of Segal *et al.*[1] and of Alberty,[2] which deal with the the kinetics of enzyme systems involving two substrates.

Further, the brilliant achievement of Vennesland and Westheimer and their collaborators[3, 4] in demonstrating that dehydrogenases catalyze a direct and stereospecific transfer of hydrogen between DPNH and the oxidized form of the substrate, gave us a clear indication that an enzyme-coenzyme-substrate complex was the reactive intermediate in such reactions and thus markedly limited the range of choice of reaction mechanisms. On the basis of this finding, the following schemes could be regarded as possible mechanisms for the enzyme-catalyzed reaction:

I

$$\begin{aligned}
&E + O \rightleftharpoons EO\\
&E + L \rightleftharpoons EL\\
&EO + L \rightleftharpoons EOL\\
&EL + O \rightleftharpoons EOL\\
&EOL \rightleftharpoons ERP\\
&ERP \rightleftharpoons ER + P\\
&ERP \rightleftharpoons EP + R, \text{ etc.}
\end{aligned}$$

II

$$\begin{aligned}
&E + O \underset{2}{\overset{1}{\rightleftharpoons}} EO\\
&EO + L \underset{4}{\overset{3}{\rightleftharpoons}} EOL \underset{6}{\overset{5}{\rightleftharpoons}} ERP \underset{8}{\overset{7}{\rightleftharpoons}} ER + P\\
&ER \underset{10}{\overset{9}{\rightleftharpoons}} E + R
\end{aligned}$$

III

$$\begin{aligned}
&E + O \rightleftharpoons EO\\
&EO + L \rightleftharpoons ER + P\\
&ER \rightleftharpoons E + R
\end{aligned}$$

In these schemes E is the enzyme, O and R are the oxidized and reduced forms of diphosphopyridine nucleotide, respectively, and L and P are lactate and pyruvate, respectively.

Case I is for the situation in which the enzyme is postulated to have separate binding sites for substrate and coenzyme, so that either coenzyme or substrate can combine with the enzyme to form a binary complex. This binary complex then combines with the other reactant to form the ternary complex EOL. The initial reaction velocity from the DPN-lactate side of the reaction is given by:[1, 2]

$$V_f/v = 1 + K_O/O + K_L/L + K_{OL}/OL \tag{1}$$

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in which V_f is the maximum reaction velocity of the forward reaction when the enzyme is saturated with respect to both DPN and lactate; K_O and K_L are the Michaelis constants for DPN and lactate, respectively; and O and L are the initial concentrations of these reactants. K_{OL} is a complex constant whose meaning depends upon the assumptions made concerning the mechanism. If it is assumed that the two binding sites for coenzyme and substrate are wholly independent, $K_{OL} = K_O K_L$. On the basis of early measurements, we concluded that this was the case for lactic dehydrogenase (LDH),[5] as it appears to be for yeast alcohol dehydrogenase (ADH),[6, 7] but more precise measurements indicated that K_{OL} was not equal to the product $K_O K_L$.[8] Therefore, the alternative possibility that the binding of one reactant results in a binary complex that has a different affinity for the second reactant than does the enzyme itself, required consideration as a possible mechanism for the LDH-catalyzed reaction.

The initial reaction velocity from the pyruvate-reduced diphosphopyridine nucleotide (DPNH) side of the reaction is given by an equation that is symmetrical with EQUATION 1.

Case II represents the hypothetical situation in which there is a compulsory order of binding of the two reactants. As written, this mechanism implies that there is no binding site on the enzyme for lactate, but that this site is generated when enzyme and DPN form a binary complex. The reverse situation, in which the substrate must be bound first and the coenzyme can be bound only by the EL complex, is also a logical possibility.

Case III represents the situation in which ternary complexes have such short half lives that they have no measurable influence upon the kinetics of the reaction. This scheme was used by Theorell and Chance[9] to account for kinetic measurements based upon the rate of disappearance of the enzyme-reduced diphosphopyridine nucleotide (DPNH) complex of liver ADH in the presence of aldehydes. Although these authors pointed out that their measurements indicated only that ternary complexes had no *kinetic* significance in their system, their results have been interpreted by other workers to mean that ternary complexes have no existence.[10]

The initial reaction velocities for cases II and III are given by equations identical in form with EQUATION 1 so that no choice between these alternate possibilities can be made on this basis. Alberty[2] pointed out that the relation between the kinetic constants, measured for each side of the reversible reaction, and the equilibrium constant for the reaction is also the same for all three cases and is given by:

$$K = (R)(P)/(O)(L) = V_f K_{RP}/V_r K_{OL} \tag{2}$$

where (R), (P), etc., are the equilibrium concentrations of products and reactants, V_r is the maximum velocity in the reverse direction of the enzymatically catalyzed reaction, and K_{RP} is the constant corresponding to K_{OL}, but for the reaction measured from the DPNH-pyruvate side. Alberty[2] further found that, in addition to satisfying EQUATION 2, case III must also satisfy the relation:

$$K = V_f^3 K_R K_P / V_r^3 K_O K_L \tag{3}$$

Table 1[8]

Temperature	Observed equilibrium constants	Equilibrium constants calculated from:	
		V_fK_{RP}/V_rK_{OL}	$V_f^3K_RK_P/V_r^3K_OK_L$
°C.			
16	0.978×10^{-5}	1.15×10^{-5}	5.29×10^{-5}
25	1.74	1.81	5.66
35	3.24	2.85	6.26

Table 1 shows the fit of equilibrium constants calculated by substitution of measured kinetic constants into equations 2 and 3 to measured equilibrium constants for a range of temperatures. The kinetic and equilibrium constants were evaluated in 0.05 M phosphate buffer, *p*H 6.80.

Although the equilibrium constants calculated by equation 2 agree well with the observed equilibrium constants, the agreement between values calculated by equation 3 is much less both as regards absolute magnitude and the variation with temperature. Case III was, therefore, dropped from further consideration.

A choice could be made between the situations represented by cases I and II if it could be shown that the enzyme bound equal mole numbers of each of the substrates and of DPN and DPNH or, alternatively, if it could be shown that coenzymes were bound and that substrates were not bound by the enzyme. The possibility that the enzyme might bind substrates but not coenzymes was ruled out by the fact that Chance and Neilands[11] had detected an LDH-DPNH complex by spectrophotometric methods.

Binding measurements were carried out using the ultracentrifugal separation technique of Velick, Hayes, and Harting.[12] The results of experiments designed to measure the binding of DPN are shown in figure 1. The plot used is that of Scatchard[13] in which r, the number of moles of DPN bound by one mole of enzyme, is plotted against the ratio of r to the equilibrium concentration of DPN. The intercept on the r axis, 3.6 ± 0.4, is the maximum number of binding sites for DPN and the negative slope, $3.9 \pm 0.7 \times 10^{-4}$ M, is the intrinsic dissociation constant of the LDH-DPN complex.[14] The lack of curvilinearity of the plot may be taken as an indication that the binding sites are essentially independent. Since there are approximately four binding sites for DPN, it is of interest that there are approximately four sulfhydryl groups that react at a measurable rate with *p*-chloromercuribenzoate (PCMB).[14]

Similar attempts to measure the binding of pyruvate or lactate by LDH yielded negative results. The supernatant solution remaining after the enzyme had been removed in an ultracentrifugal field actually contained pyruvate or lactate at concentrations slightly higher than those of the starting solutions. Since the enzyme preparation contained no detectable substrate, this finding was accounted for on the basis that removal of the enzyme protein, which fills space but which is not a solvent for pyruvate or lactate, left a more concentrated solution of these materials in the supernatant solutions.[14]

On the basis of these findings, kinetic case I was dropped from consideration,

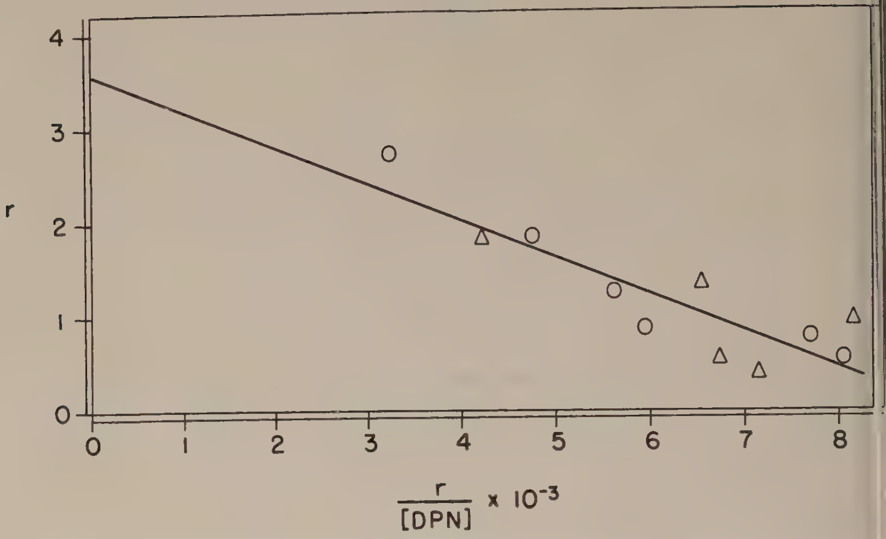

FIGURE 1. Mode of action of heart muscle lactic dehydrogenase. Reproduced by permission of *The Journal of Biological Chemistry.*

and the remaining work has been framed in the concept of case II. Although the steady-state derivation of EQUATION 1 leads to definitions of the kinetic constants which are quite complex, simple relationships exist between some of these constants.[14] For the constants describing initial reaction velocities from the lactate-DPN side of the reaction,

$$K_{OL}/K_L = k_2/k_1 \tag{4}$$

$$V_f/K_O = k_1 \tag{5}$$

and

$$K_{OL}V_f/K_LK_O = k_2 \tag{6}$$

where k_1 and k_2 are the rate constants for the correspondingly numbered reactions in kinetic case II. Similar relations apply to the constants for the reverse reaction.

The remaining major kinetic problem appeared to be the assignment of a role to the proton which appears as a stoichiometric reactant in the reversible reaction:

$$\text{Lactate} + \text{DPN}^+ \rightleftharpoons \text{Pyruvate} + \text{DPNH} + \text{H}^+ \tag{7}$$

This problem was approached by determining the variation of the various kinetic parameters with pH over the range from pH 5.5 to 10.5.[15] Since pronounced anion effects are found when one buffer is substituted for another, Tris-(hydroxymethyl)aminomethane buffers made up to a constant ionic strength of 0.2 with KCl were used over the entire pH range. The variation of the various kinetic parameters as a function of pH is shown in FIGURES 2 to 5.

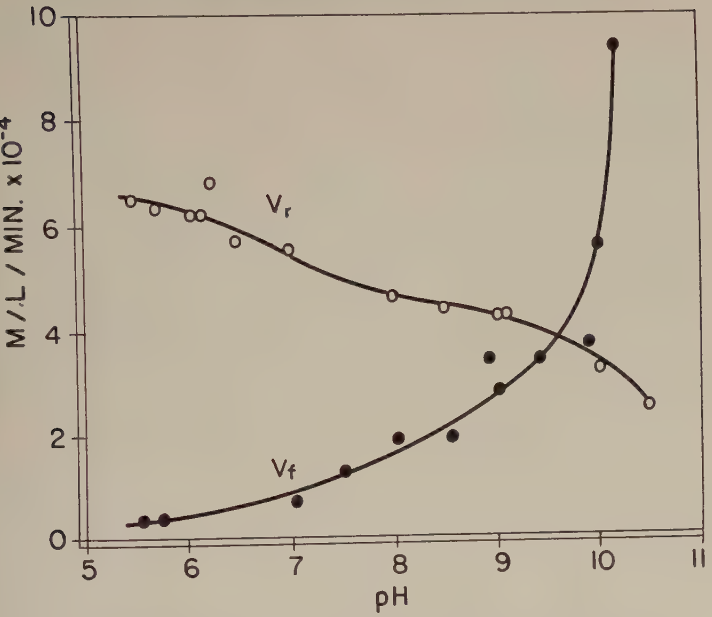

FIGURE 2. Mode of action of heart muscle lactic dehydrogenase

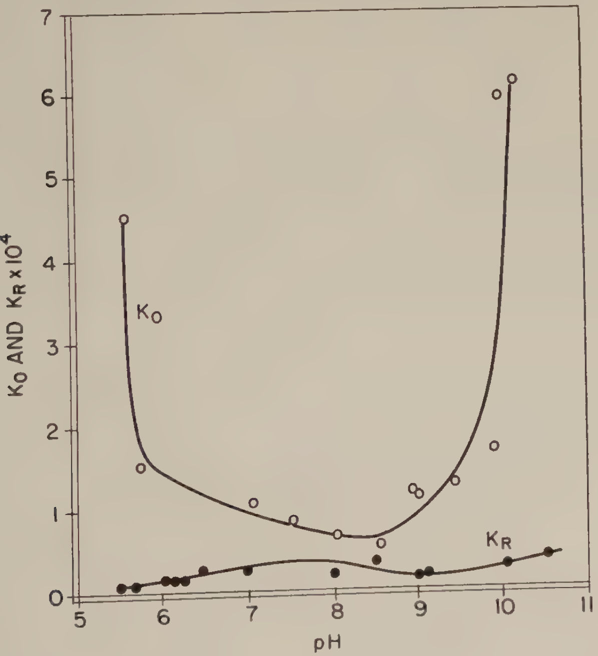

FIGURE 3. Mode of action of heart muscle lactic dehydrogenase

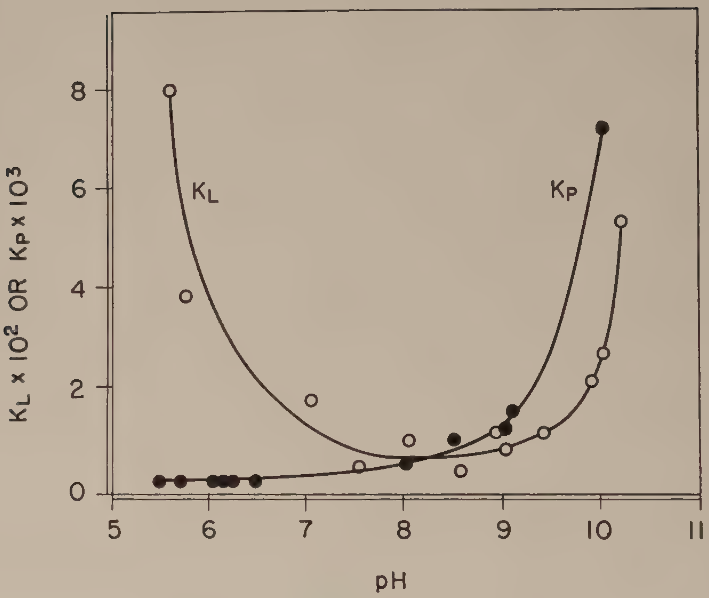

FIGURE 4. Mode of action of heart muscle lactic dehydrogenase

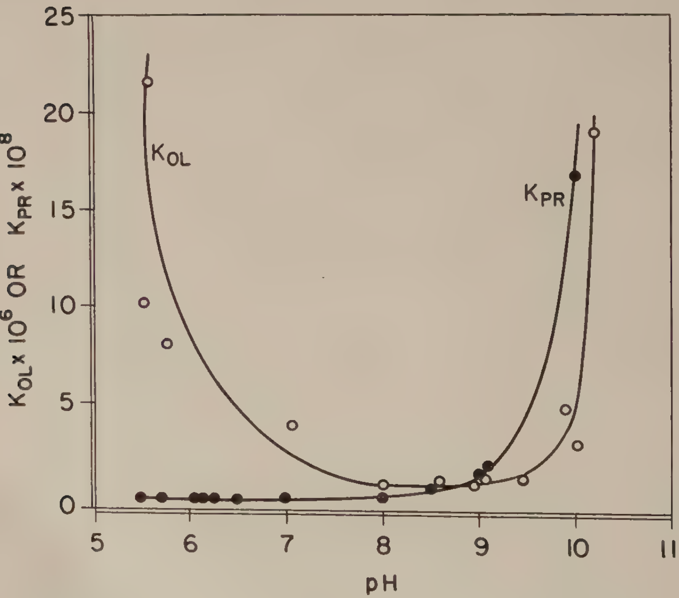

FIGURE 5. Mode of action of heart muscle lactic dehydrogenase

Since the evaluation of the kinetic parameters is subject to considerable experimental and mathematical error, it was desirable to test the results for consistency and accuracy before attempts were made at interpretation. Internal consistency may be tested by determining whether substitution of the kinetic parameters into EQUATION 2 agrees with the variation with *p*H of the observed equilibrium constant predicted from EQUATION 7. Since the difference between the predicted value of K and the value calculated from EQUATION 2 amounts to a factor of approximately 5 when the hydrogen ion concentration is varied by a factor of nearly 100,000, the results appear to be reasonably consistent.

EQUATION 4 and its analogue for the reverse reaction permit the estimation of the dissociation constants for the dissociation of EO and ER complexes into LDH and DPN or DPNH, respectively. The values of these dissociation constants, calculated in this way, were checked by two independent methods. The first of these depends upon the observation that, in the presence of either DPN or DPNH, the enzyme is protected against PCMB inactivation. Since the rate of PCMB inactivation follows first-order kinetics when PCMB is in excess, the first-order rate constant for the inactivation will be diminished in direct proportion to the fraction of the enzyme which exists as EO or ER when DPN or DPNH is added to the system. Since the fraction of the enzyme existing as EO or ER complex and the concentration of added DPN or DPNH are known, the dissociation constants of the binary complexes can be estimated.

An alternate approach to the same problem is provided by the observation that DPN and DPNH compete for the same site on the enzyme surface. Thus, if DPNH is added to a system containing LDH, DPN, and lactate, the added DPNH acts as a competitive inhibitor. K_I, estimated by conventional methods, is clearly the dissociation constant of the ER complex. A comparison of dissociation constants estimated by these techniques with those estimated from the use of the kinetic parameters is shown in TABLE 2.

In view of the nature of the measurements, this order of agreement appears to be satisfactory. The impression, gained from the use of kinetic parameters alone, that the dissociation of the EO complex is relatively insensitive to *p*H

TABLE 2[15]

VALUES OF DISSOCIATION CONSTANTS OF ENZYME-COENZYME COMPLEXES DETERMINED BY DIFFERENT METHODS

*p*H	Dissociation constant of EO complex from:			Dissociation constant of ER complex from:		
	K_{OL}/K_L	Protection against PCMB	Rate inhibition by DPN	K_{RP}/K_P	Protection against PCMB	Rate inhibition by DPNH
	10^{-4} *M*	10^{-4} *M*	10^{-4} *M*	10^{-6} *M*	10^{-6} *M*	10^{-6} *M*
6.80	1.8	—	2.7	3.8	—	2.2
7.01	1.8	2.2	—	—	—	—
8.03	2.0	1.4	—	2.5	2.2	—
8.58	1.7	—	1.0	7.8	—	6.3
9.85	1.6	—	3.5	—	—	—
10.02	—	—	—	23.0	20.0	—

changes, while the *ER* complex dissociates more readily at high *p*H values, is confirmed by the independent measurements.

By making use of EQUATIONS 5 and 6 and of the analogous equations for the reverse reaction, the variation of k_1, k_2, k_9, and k_{10} with *p*H can be estimated. Model mechanisms can then be tested for fit to these values. A mechanism which gives satisfactory fit to the values for k_9 and k_{10} is shown in FIGURE 6.[15]

This mechanism assumes that two ionizable groups on the protein surface are involved in the binding of DPNH to the LDH surface. When both groups are protonated, DPNH is bound firmly, the dissociation constant of the $^{+}E^{+}R$ complex being approximately 8×10^{-7} M; when the more strongly acidic group dissociates as the *p*H is raised, the dissociation constant of the resulting complex is approximately ten times larger; and when the second group dissociates, the dissociation constant is increased by an additional factor of approximately 500. According to this scheme, the dissociable group in the $^{\circ}E^{+}R$ complex is a much weaker acid than the same group on the surface of the free enzyme. This effect stabilized the $^{\circ}E^{+}R$ complex and may account for relatively slow rate of change of maximal velocity of the reverse reaction with *p*H (FIGURE 2).

The values obtained for k_9 are in reasonable agreement with the values observed for V_f so that it is probable that the limiting velocity in the conversion of DPN and lactate to DPNH and pyruvate is the dissociation of the LDH-DPNH complex. Theorell and Chance[9] arrived at a similar conclusion by a wholly different experimental method in studies of liver ADH.

The observation that the stability of the LDH-DPN complex is relatively independent of *p*H, taken together with the pronounced minima in the plots of K_O, K_L, and K_{OL} against *p*H (FIGURES 3 to 5), has led to the scheme shown in FIGURE 7 for the interaction of LDH and DPN. It is again assumed that there are two dissociable groups on the enzyme surface but, for this side of the reaction, the results seem to indicate that the binding of DPN to the LDH surface is independent of the charge state of these groups. Lactate, however, is bound only by that form of the LDH-DPN complex in which the more

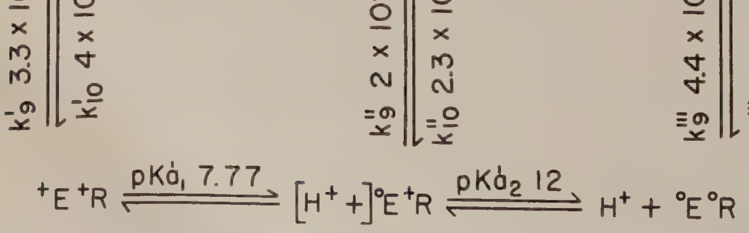

FIGURE 6. Mode of action of heart muscle lactic dehydrogenase. Reproduced by permission of *The Journal of Biological Chemistry.*

strongly acidic group is unprotonated and the less acidic group is protonated. The experimental results are fitted by assigning pK values of 7.7 and 9.7 to the two groups.

The similarity of the pK values deduced from the results for the forward and reverse reactions suggests that the same ionizable groups are involved. The group that has a pK value in the region of pH 7 has been taken to be the imidazolium group of histidine. The group with a pK value of approximately 9.7 could be the ϵ-ammonium of lysine or a sulfhydryl group. On the basis of the protection afforded the enzyme by either DPN or DPNH against PCMB inactivation, this group has been considered to be a sulfhydryl group.

Taken together, the results derived from kinetic measurements of each side of the reversible reaction suggest strongly that a specific imidazolium group on the enzyme surface is the source and sink of the proton involved in the reaction. The oxidation of lactate requires that this group be unprotonated so that it can accept the stoichiometric proton; conversely, the reduction of pyruvate appears to require that this group be protonated. The latter point is based chiefly on inference, since the kinetic results yield no information concerning the mode of binding of pyruvate to the LDH-DPNH complex. It has been found, however, that oxamic acid, the half amide of oxalic acid, is a powerful inhibitor of the enzymatic reaction and, further, that oxamate is competitive with pyruvate.[16, 17] If the K_I value for oxamate is measured over the range of pH which was used for the kinetic studies, it is found that K_I varies with pH in essentially the same way as does K_P (FIGURE 4).[18] If it is assumed that oxamate is bound to the LDH-DPNH surface by the same forces that bind pyruvate to this surface, it follows that the complex designated as $^+E^+R$ in FIGURE 6 has the greatest affinity for pyruvate, and that the $^+E^+RP$ complex is the active complex from the pyruvate-DPNH side of the reaction.

If it is accepted that the reduction of pyruvate by DPNH involves the

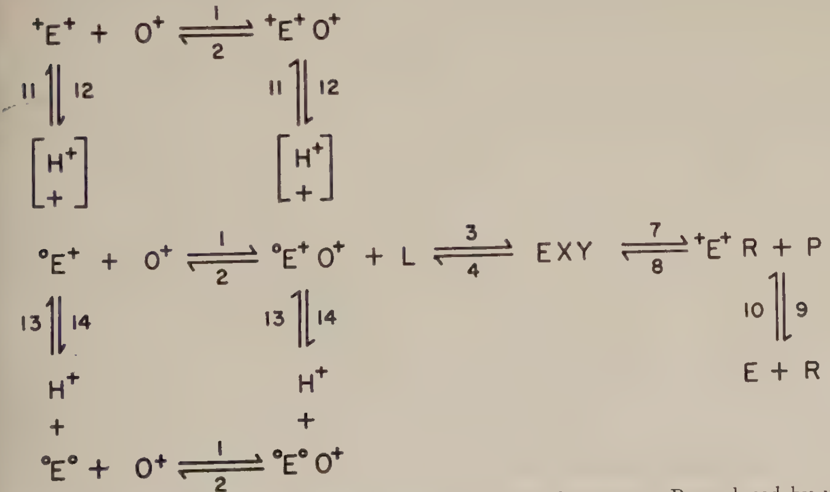

FIGURE 7. Mode of action of heart muscle lactic dehydrogenase. Reproduced by permission of *The Journal of Biological Chemistry*.

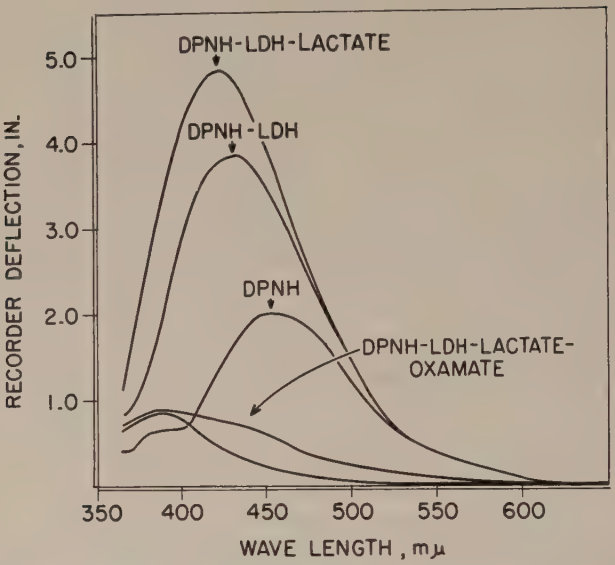

FIGURE 8. Mode of action of heart muscle lactic dehydrogenase. Reproduced by permission of *The Journal of the American Chemical Society.*

transfer of a proton from imidazolium ring on the LDH surface to the carbonyl oxygen of pyruvate, it seems logical to assume that the rest of the reduction involves the transfer of a hydride ion from the *para*-position of the nicotinamide to the *alpha*-carbon of pyruvate. Alfred D. Winer has recently made observations that suggest that the LDH-DPNH complex reacts with oxamate in a similar manner but that, since no transfer of electrons from DPNH to oxamate can occur, the result is an abortive, stable activated LDH-DPNH-oxamate complex.[19]

This conclusion was based on the finding illustrated in FIGURE 8, that the fluorescence spectrum of DPNH is shifted toward shorter wave lengths and greatly increased in intensity in the presence of LDH. This experiment was suggested by the earlier observation of the same effect by Boyer and Theorell[20] with liver ADH and DPNH. A similar observation has been made recently with yeast ADH and DPNH.[21] When oxamate is added to the system, the fluorescence of the complex and of DPNH itself is quenched. Although quenching effects are familiar in fluorimetry, the present effect is very probably not simple quenching since the same concentration of oxamate that reduces the velocity of the enzymatic reduction of pyruvate by one half also reduces the intensity of fluorescence of the LDH-DPNH complex by one half. Further, the addition of rather large concentrations of L-lactate increases the intensity of the LDH-DPNH fluorescence, while D-lactate has no effect on this fluorescence. Since DPN does not fluoresce, it is proposed that in the LDH-DPNH-oxamate complex the carbonyl of oxamate is stabilized in a polarized configuration by the charged imidazolium group and that, as a consequence, the hydride ion is attracted to the *alpha*-carbon of oxamate. To the extent that the hydride ion is pulled away from the nicotinamide ring, the latter assumes an aromatic structure and loses its characteristic fluorescence. This notion is admittedly highly speculative, but it is being used as a working hypothesis for the further investigation of this phenomenon.

Acknowledgments

While most of the experimental and mathematical details have been omitted from this discussion, mention of the patience and ingenuity of my past and present collaborators, Maire T. Hakala, Andrew J. Glaid, Yasuo Takenaka, Alfred D. Winer, and William B. Novoa, cannot be omitted.

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THE MECHANISMS OF ALTERATION IN LACTIC DEHYDROGENASE ACTIVITY OF BODY FLUIDS*

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Introduction

Lactic dehydrogenase (LD) is present in most animal and human tissues, as well as in body fluids such as urine, serum, serous effusion, and cerebrospinal fluid. By different mechanisms, LD activity varies in body fluids in response to pathological processes in tissues bathed by these fluids. It is the purpose of this presentation to discuss the alterations of LD activity in serum (S), serous effusion (SE), and cerebrospinal fluid (CSF) in an attempt to elucidate some of the mechanisms by which these body fluid enzyme changes occur.

The enzyme LD is concerned with the interconversion of pyruvate and lactate in the presence of diphosphopyridine nucleotide (FIGURE 1). Lactic dehydrogenase activity may be measured spectrophotometrically by determining the rate of oxidation of reduced diphosphopyridine nucleotide by pyruvate at *p*H 7.4.[1,2] One unit of LD activity has been defined as the enzyme activity present in 1.0 ml. of body fluid that results in a decrease in optical density of 0.001 per minute of a reaction mixture under specified conditions. Body fluids are examined for LD activity within 48 hours after collection and are stored at 4° C during that time interval. Hemolyzed specimens are not used, but sanguineous samples free of hemolysis in the centrifuged supernatant may be utilized. The appreciable LD activity of erythrocytes results in artifactual increments of LD of body fluids when hemolysis has occurred. Fluid samples are centrifuged at 2000 rpm for 5 minutes at 26° C., and the supernatant free of formed and undissolved elements is used for the determination of LD activity.

Mechanisms Involved in Serum Lactic Dehydrogenase Alterations

Necrosis of tissue results in the release of intracellular LD, which finds access into the extracellular fluid compartment, resulting in an increase in serum lactic dehydrogenase (SLD).[3] In experimentally produced coronary occlusion resulting in myocardial infarction in dogs, SLD increases in proportion to the amount of necrotic myocardial tissue (FIGURE 2). Other intracellular enzymes are likewise liberated into the circulation and appear in proportion to their activity in the infarcted tissue. However, the relative increase of serum enzymes is partly influenced by the normal range of activity of the serum enzyme. In instances in which the serum normally is richly active with an enzyme activity, large tissue amounts of enzyme when contributed to the extracellular fluid compartment may result in relatively smaller increments of enzyme ac-

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tivity than other enzymes of lesser tissue activity. TABLE 1 compares dog serum and cardiac tissue homogenate activity of three enzymes, including LD. FIGURE 3 contrasts the serum alterations of the same three serum enzymes following experimental myocardial infarction in the dog. It is pertinent that

Lactic Dehydrogenase

$$\text{pyruvate} + \text{DPNH} \rightleftharpoons \text{lactate} + \text{DPN}$$

FIGURE 1. Reversible reaction catalyzed by lactic dehydrogenase

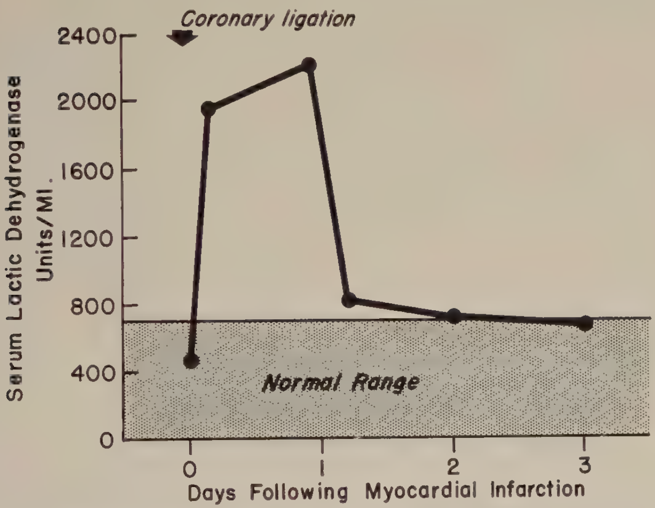

FIGURE 2. Quantitative and serial alterations of serum lactic dehydrogenase activity following experimentally produced coronary occlusion in a dog.

TABLE 1

RANGE OF ENZYME ACTIVITY IN THE DOG

Three Enzyme Activities of Serum and Cardiac Tissue Homogenates from Canine and Human Sources

Enzyme	GOT*	GPT†	LD‡
Serum... Units/ml...	5 40	5 40	100 600
Cardiac tissue homogenate... Units/gm...	150,000 400,000	20,000 80,000	300,000 700,000

* GOT: glutamic-oxaloacetic transaminase.
† GPT: glutamic-pyruvic transaminase
‡ LD: lactic dehydrogenase

SLD is only minimally increased above the normal range, although dog cardiac tissue enzyme is rich in LD activity.[4] Presumably the smallest of the three enzyme changes is reflected in the SLD alteration and is due to the greater normal range of activity of the SLD activity. Although some differences in the rate of fall of the three serum enzymes may occur, as suggested by the observations of serum enzymes after intravenous injection of dog heart homogenate (FIGURE 4), this difference alone would not account for the relatively low peak SLD value after experimental myocardial infarction. From these and other observations it would appear that SLD activity alterations associated with tissue necrosis are influenced by the amount of enzyme present in the necrotic tissue, the extent of the necrosis and the rapidity with which it occurs, the rate of loss of enzyme from the tissue and the accessibility of the lost enzyme to the circulation, and the normal range of SLD activity. No inhibiting or activating factors for SLD have been identified. Although LD activity is present in urine, renal status including oliguria and anuric states, do not appear to influence the level of SLD significantly unless necrosis of renal tissue is present.

In human diseases involving tissue necrosis, essentially the same factors are concerned with mechanisms for the alteration in SLD as were discussed for experimental settings. TABLE 2 lists results of the LD activity assay of a group of normal human tissues. For four to six days following myocardial infarction, SLD is increased above the normal range (FIGURE 5). Human

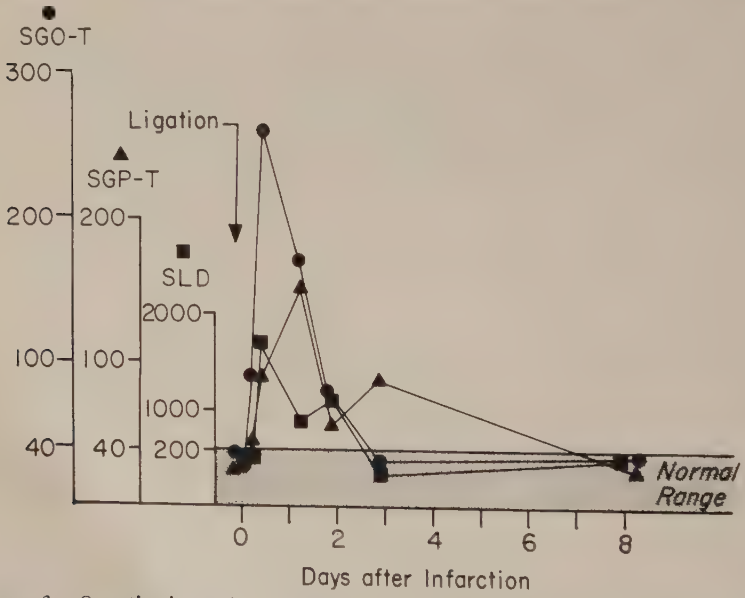

FIGURE 3. Quantitative and serial alterations of serum glutamic-oxaloacetic transaminase (SGO-T), serum glutamic-pyruvic transaminase (SGP-T), and serum lactic dehydrogenase (SLD) in dog A-204 following experimentally produced myocardial infarction from coronary artery ligation. The infarct tissue was estimated at post-mortem examination to weigh 30 gm.

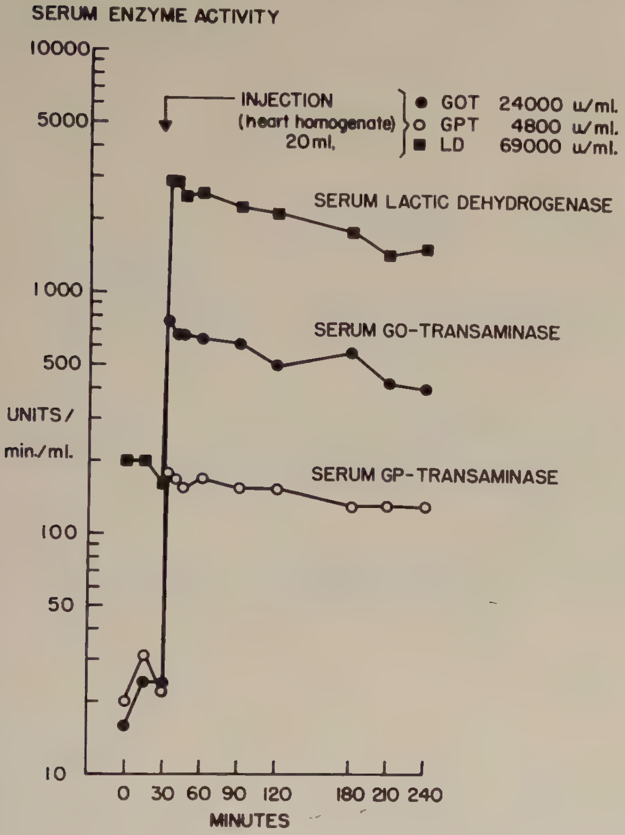

FIGURE 4. Rate of fall of three serum enzymes following the intravenous injection of dog cardiac homogenate.

TABLE 2
ESTIMATED LACTIC DEHYDROGENASE ACTIVITY OF HUMAN TISSUES
Per Gram of Homogenate

Tissue	Activity (units/gm. of wet tissue)
Kidney	640,000
Skeletal muscle	600,000
Liver	390,000
Heart	240,000
Pancreas	150,000
Spleen	140,000
Brain	130,000
Lung	25,000
Serum	400

heart tissue is rich in both LD and glutamic-oxaloacetic activity and is released similarly during acute myocardial infarction (FIGURE 6). In skeletal muscle trauma and/or surgical trauma resulting in tissue necrosis, necrotizing pancreatitis, and fulminant hemolytic states, SLD is similarly increased. However, in inflammatory-necrotic diseases of organs rich in LD, SLD is usually increased minimally or not at all. In the course of homologous serum hepatitis, huge increments in serum glutamic-oxaloacetic transaminase are seen with only minimal SLD alterations; these serum enzyme changes occur, although the liver is richer in LD than human heart tissue whereas glutamic-oxaloacetic transaminase is present more abundantly in human heart tissue than in hepatic tissue. Pathological states involving inflammatory factors are accompanied by SLD alterations influenced in large part by factors other than tissue enzyme activity. What these other factors are is unclear at present, but it would appear that inflammatory processes may selectively alter intracellular enzyme permeability, as well as the mechanisms for serum enzyme degradation.

SLD elevations have been noted in experimentally induced leukemia and in transplantable carcinoma and sarcomas in rodents (FIGURE 7, TABLES 3 and 4).[5, 6] These alterations occur in the absence of demonstrable tissue necrosis, and they appear, in the case of some malignant mouse tumors, within 6 to 48 hours after transplantation of tumor tissue (FIGURE 8); the LD content of the

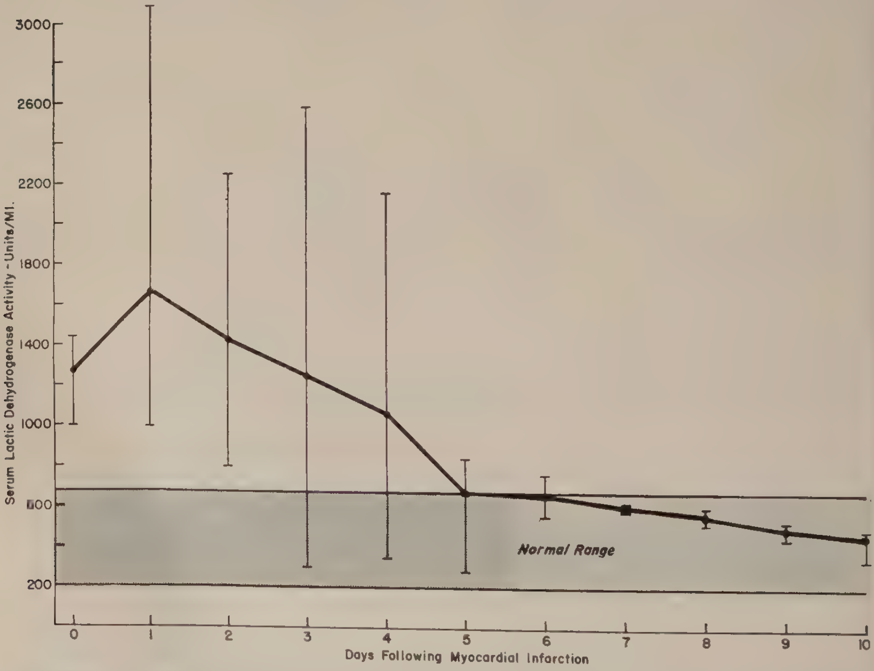

FIGURE 5. Mean serum lactic dehydrogenase activity on 10 consecutive days in 20 patients who incurred acute transmural myocardial infarction. The top and bottom of the vertical lines represent the highest and lowest serum lactic dehydrogenase activity noted.

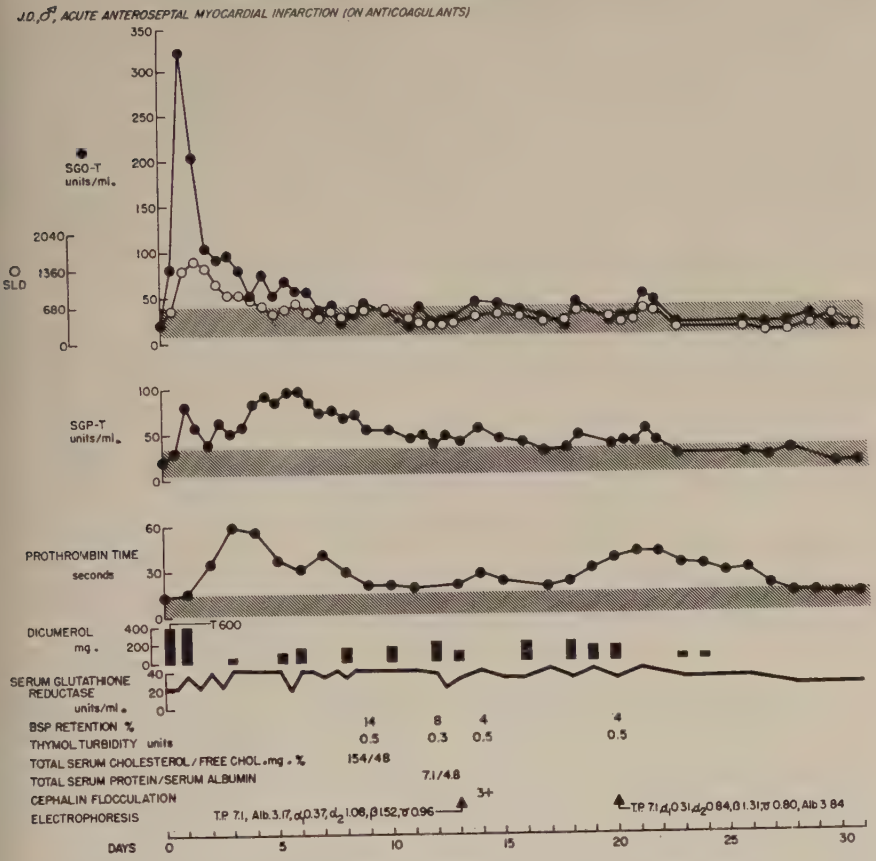

FIGURE 6. Serial alterations of serum glutamic-oxalaocetic transaminase, serum lactic dehydrogenase, and other laboratory parameters in a male patient who incurred an acute transmural myocardial infarction.

transplanted malignant tissue is less than 200 units and could not account for the SLD alterations noted. In addition, the transplantation of normal tissue such as normal mouse liver and spleen results in no significant increment in SLD. Simultaneous estimation of tumor tissue LD, hepatic tissue LD, and SLD during the course of sarcoma 180 suggests that the SLD increment may be a reflection of increased hepatic and/or tumor release of LD (FIGURE 8). The rapid growth of nonmalignant tissue does not result in increased SLD as evidenced by the fact that following subtotal hepatectomy in mice, the regeneration of the liver to normal size occurs within 10 to 12 days and is accompanied by no significant SLD alterations beyond the normal range (TABLE 5). Pregnancy in mice is accompanied by small elevations of SLD, although no significant alterations of SLD have been observed during the course of human pregnancy.

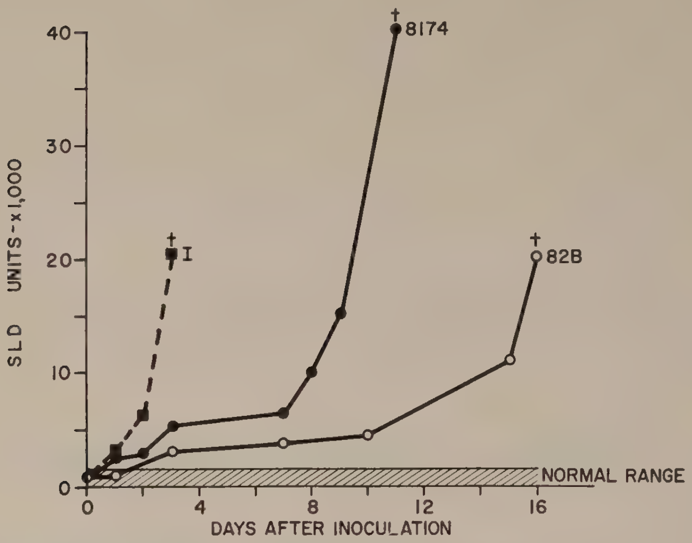

Figure 7. Serial alterations of serum lactic dehydrogenase activity in various virus (transplantable) leukemias of F_1 (C58 by Balb) mice.

Table 3
Transplantable Tumors in Mice

Tumor	Days following implantation	Plasma lactic dehydrogenase
		units/min./.01 ml.
Ehrlich's carcinoma	15	163
Ehrlich's carcinoma	20	258
Bashford carcinoma 63	14	186
Bashford carcinoma 63	17	118
Adenocarcinoma E 0771	14	240
Adenocarcinoma E 0771	21	180
Myomo adenocarcinoma	22	120
Lewis bladder carcinoma	14	90
Lewis bladder carcinoma	15	160
Normal mice		14–4.0

Table 4
Transplantable Tumors in Rats

Tumor	Days following implantation	Plasma lactic dehydrogenase
		units/min./.01 ml.
Flexner-Jobling carcinoma	28	21
Walker carcinosarcoma	21	45
Jensen sarcoma	15	40
Murphy-Sturm lymphosarcoma	20	45
Normal rats		8.4–3.1

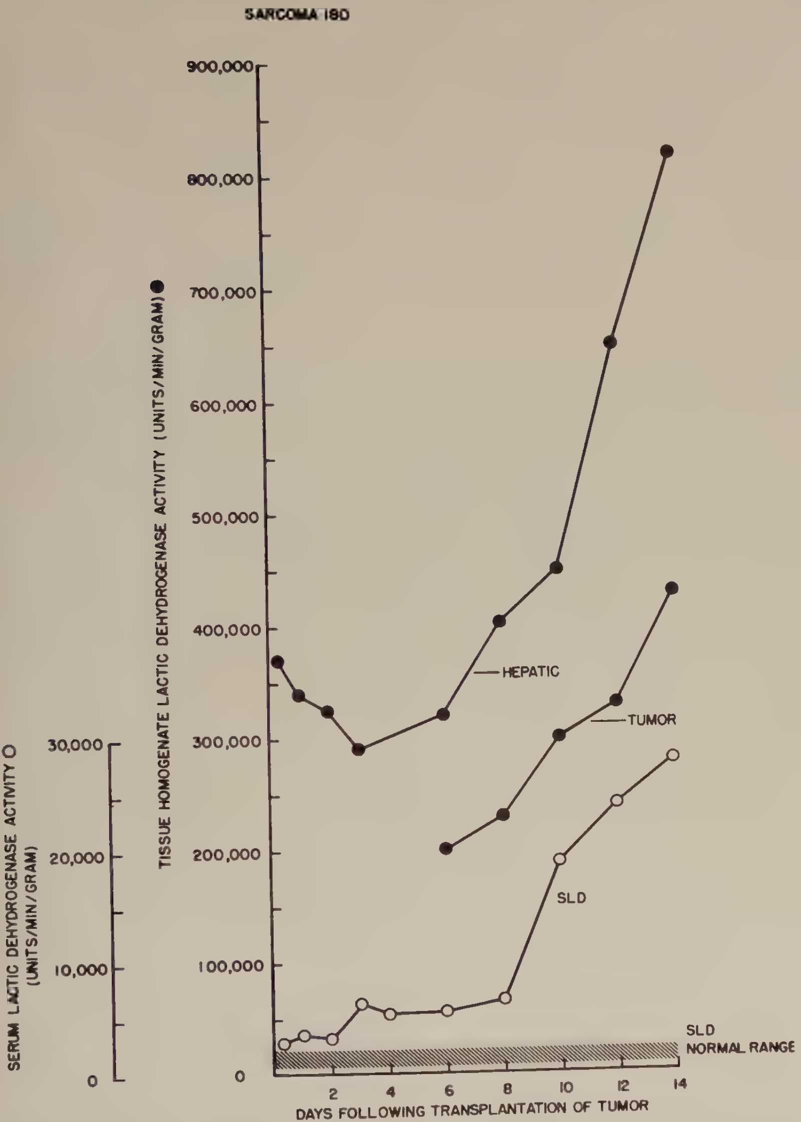

FIGURE 8. Serial alterations of lactic dehydrogenase activity of hepatic tissue homogenate, tumor tissue homogenate, and serum of mice with transplantable sarcoma 180. The hatched area represents the normal range of serum lactic dehydrogenase activity of the control mice.

One of the mechanisms for increased SLD in both experimental animals and in humans with malignant neoplasia in the absence of sustained tissue necrosis is suggested by observations of LD alterations in tissue culture fluids.* Benign

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* A. E. Moore and Felix Wróblewski, unpublished observations.

TABLE 5
SUBTOTAL HEPATECTOMY
(Average of three mice each)

Day following hepatectomy	Serum lactic dehydrogenase
	units/ml./min.
Third	1300
Sixth	1200
Ninth	1000
Twelfth	1200
Normal mice	1400 ± 400

tissues, both of rodent and human origin, are associated with minimal increments in LD activity of the bathing medium. However, malignant tissues in culture are accompanied by large increments in LD activity of the fluid medium. The elaboration of LD under conditions described in tissue culture occurs in the absence of cellular necrosis and is not accompanied by other enzyme changes studied. It appears that the growth of cells in tissue culture is accompanied by elaboration of LD in the absence of necrosis and that malignant tissue culture growth is accompanied by large fluid medium LD activity (FIGURE 9). Extension of these tissue culture studies suggest that the elaboration of LD is not only a function of cell growth, but more probably in part a reflection of the disturbed metabolic activity of malignant cells.

Although different disease states produce quantitatively similar SLD alterations, it appears that different mechanisms contribute to the serum enzyme

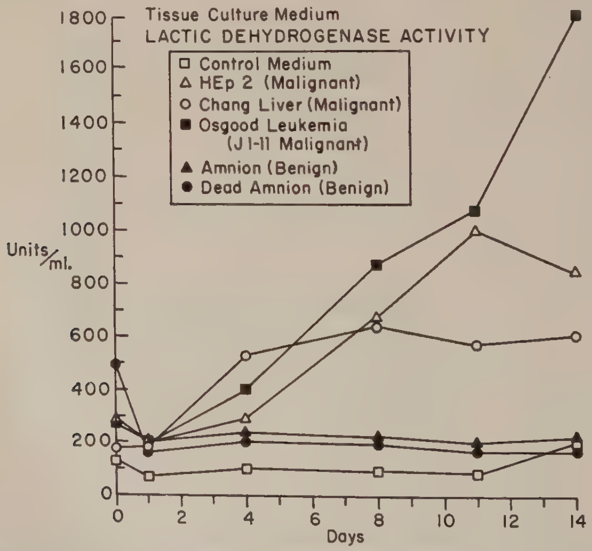

FIGURE 9. Serial alterations of lactic dehydrogenase activity of the media from various benign and malignant tissues in culture.

increments. In situations where tissue necrosis occurs, intracellular LD is released into the circulation. In inflammatory states involving organs rich in LD, little or no SLD alterations may occur unless cellular necrosis accompanies the inflammatory process. When both inflammatory and necrotic factors are present, SLD activity may be elevated, but not necessarily in proportion to the tissue enzyme concentration. When SLD alterations accompany malignant neoplasia in the absence of necrosis, it would appear that the circulating enzyme may be derived in part from the enzyme elaborated by the growth of the malignant tissue and/or the production of LD by the liver. Undoubtedly other mechanisms account for SLD alterations in these and other disease states, but as yet these factors are undefined and obscure.

Mechanisms Involved in Serous Effusion Lactic Dehydrogenase Alterations

Pericardial, pleural, and peritoneal effusions may be considered as *in vivo* cultures bathing mesothelial or malignant cells. The protein of the effusion is derived from the plasma, and the effusion has a lower protein content than the plasma. Similarly, the LD activity of a serous effusion would be expected to be lower than the plasma. In instances of serous effusions free of malignant cells both from patients with malignant neoplastic disease and with other than neoplastic disease, SE-LD is less than the serum LD activity. If malignant cells in tissue culture contribute LD activity to the bathing medium, then it is to be expected that serous effusions either containing malignant cells or bathing malignant tissue would be enriched with LD derived from the growing malignant cells. Inasmuch as LD is a globulin and serous effusion, globulins come into equilibrium with the serum globulins slowly; the LD activity of the effusion tends to accumulate in the relatively loculated effusion and gradually reaches levels greater than that of the serum of the same individual.

In Ehrlich's ascites carcinoma, the ascitic fluid contains malignant cells; the SE-LD is greater than the SLD of the same mouse. In human pleural effusions containing malignant cells, the SE-LD is greater than the respective SLD (FIGURE 10).[7] Similarly, in pericardial effusions containing malignant cells, SE-LD is greater than the LD of the plasma from which the effusion is derived (FIGURE 11). TABLE 6 shows the LD activity of serial serum and effusion samples from a patient with uterine adenocarcinoma with peritoneal metastases. In instances in which the peritoneal surface is involved by malignant tumor that does not exfoliate cells into the bathing effusion, the LD relationship of serum to effusion may serve to supplement negative cytological examination of a peritoneal effusion (TABLE 7). The generalization in regard to SE-LD activity and SLD and the presence or absence of malignant neoplastic tissue in the serous cavity is not valid if hemolysis is present in either the serum or effusion examined or if the effusion is purulent or chylous. The other limitations of the technique have been described previously.

Mechanisms Involved in Cerebrospinal Fluid Lactic Dehydrogenase Alterations

In the absence of central nervous system disease, LD activity of cerebrospinal fluid (CSF) varies between 5 to 40 units per ml. with no significant difference in activity between fluid obtained by spinal tap between the fourth and fifth lumbar vertebrae and fluid removed from the lateral ventricles. When the blood-brain barriers are intact, CSF-LD is independent of the tenfold greater LD activity of the plasma; variations of plasma LD activity are not reflected

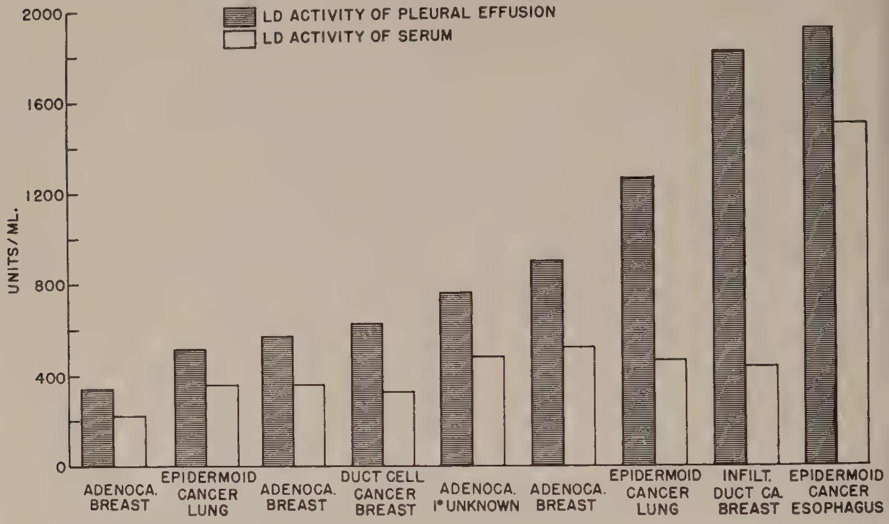

FIGURE 10. Lactic dehydrogenase activity of serous effusion and serum obtained from patients with malignant neoplastic diseases. The serous effusions all contained malignant cells.

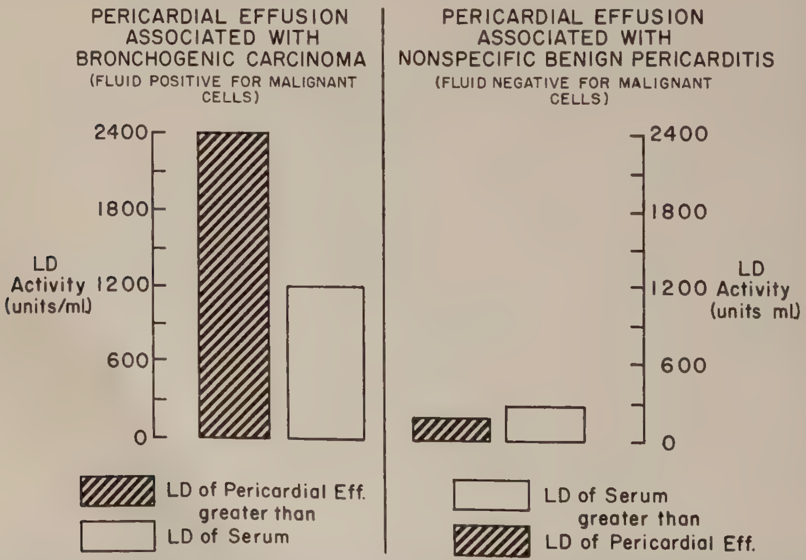

FIGURE 11. Lactic dehydrogenase activity of pericardial effusion and serum from two patients. One effusion contained malignant cells; the other was free of malignant cells.

TABLE 6

UTERINE ADENOCARCINOMA (GRADE 3) PERITONEAL METASTASES WITH ASCITES

Laboratory Data Derived from Examination of Peritoneal Effusions Obtained from a Patient (S. M., 77 Years) with Uterine Adenocarcinoma

Hospital day	LD activity units per ml.		Peritoneal fluid cytologic examination for malignant cells (smear and cell block)
	Serum	Effusion	
6	800	1200	Negative
7	1000	1300	Positive*
11	2000	4700	
12	(Intraperitoneal radioactive gold)		
18	1700	3300	Positive (class V)
25	2400	3000	Positive (class V)
31	Expired		

* Sp. gr. 1.015 protein 3.7 gm per cent.

TABLE 7

WILMS'S TUMOR, LEFT KIDNEY METASTASES TO PERITONEAL SURFACE OF DIAPHRAGM, PARIETAL PLEURA, HEART AND LUNGS

Laboratory Data Derived from Examination of Peritoneal Effusions Obtained from a Patient (S.P., 7 Years, Male) with Wilms's Tumor of Left Kidney

	Ascites				Serum LD	Effusion LD
	Volume	Color	Protein	Cytology		
	(ml.)		*Gm.%*		*Units/ml.*	
4/11/57	2700	Sang.	3.9	Neg.	1600	2000
5/20/57	3200	Sang.	3.7	Neg.	2200	3400

Although no malignant cells were seen in the peritoneal fluid, the lactic dehydrogenase activity of the effusion was greater than that of the serum. Post-mortem examination (May 23, 1957) revealed metastatic implants on the peritoneal surfaces bathed by peritoneal fluid.

in alterations in CSF-LD. In three groups of pathological states, CSF-LD is increased beyond the normal range: (1) acute cerebrovascular diseases, (2) acute meningitis, and (3) primary and metastatic malignant neoplasia of the central nervous system.[8] Different mechanisms appear to account for the CSF-LD alterations noted in the various disease states mentioned.

Acute cerebral thrombosis and cerebrovascular emboli result in areas of cerebral infarction. Brain tissue contains per gram about one third the LD present in liver. Accordingly, the infarcted area may lose LD and contribute the enzyme to CSF in an amount proportional to the amount of the tissue infarcted. In the absence of further insults to the central nervous system, the CSF-LD gradually decreases from a maximum activity within 24 to 72 hours to a normal range within 5 to 10 days (FIGURE 12). Cerebral hemorrhage without bleeding into the subarachnoid space may occur without increase in CSF-LD or with increments of CSF-LD up to 75 to 100 units/ml.; these enzyme alterations may represent enzyme contributions from infarcted and/or necrotic brain tissue and/or from degradation of extravasated blood. Cerebral

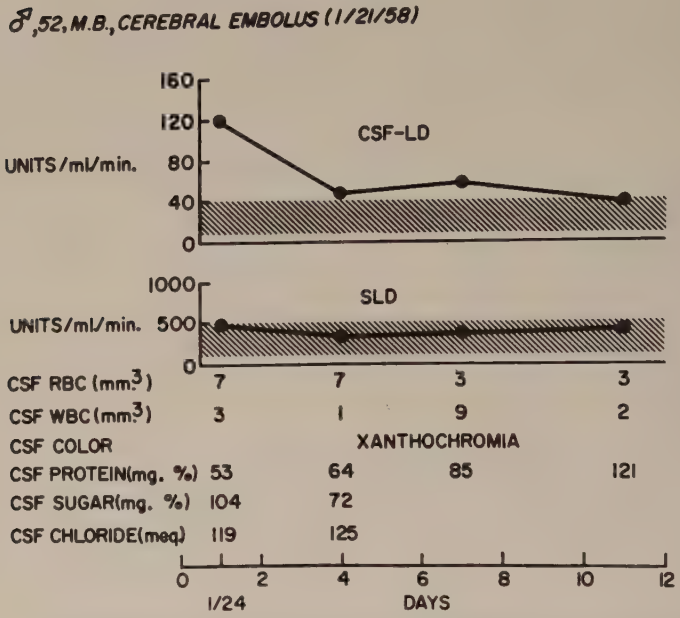

FIGURE 12. Cerebrospinal fluid and serum lactic dehydrogenase activity and other laboratory parameters observed in a patient with cerebral infarction.

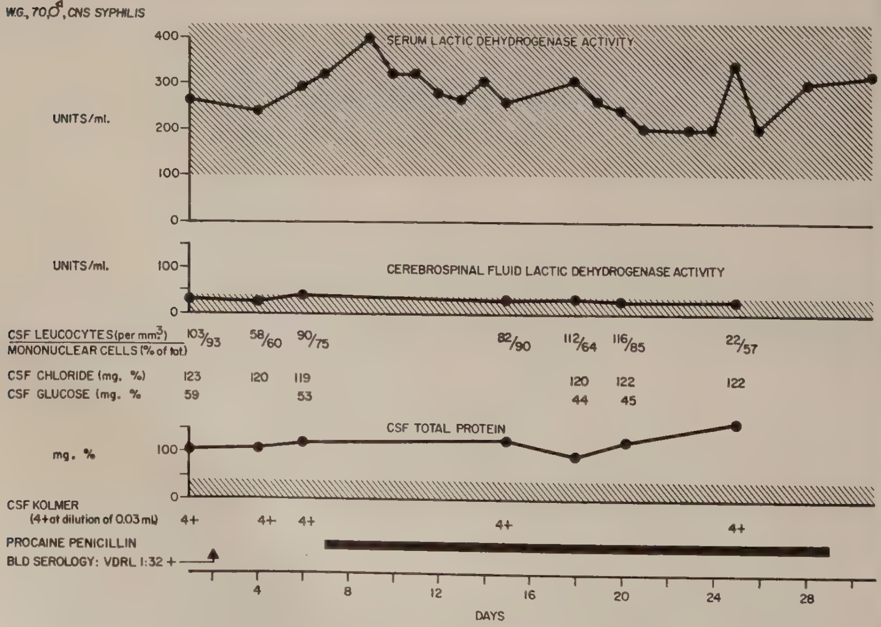

FIGURE 13. Lactic dehydrogenase activity and other laboratory parameters of serum and cerebrospinal fluid obtained from a patient with active central nervous system syphilis. A post-mortem examination confirmed the diagnosis and revealed nutritional cirrhosis.

hemorrhage, which communicates with the subarachnoid space, results in sizable increments of CSF-LD, in large part due to the contribution of plasma and erythrocyte LD activity, which are approximately 10 to 1000 times greater, respectively, than CSF-LD activity. The changes in CSF-LD activity in cerebrovascular diseases cannot be correlated with the presence of xanthochromia, numbers of leukocytes or erythrocytes, chloride, glucose, or total protein content, or the serologic reactions of the CSF (FIGURE 13).

Acute bacterial meningitis is associated with impressive increments in CSF-LD. With control of the meningeal infection, the CSF-LD returns to the normal range of activity (FIGURE 14). The presence of meningeal inflammation compromises the blood-brain barrier, presumably at the site of the meningeal vascular supply, thereby resulting in a release of leukocytes and plasma proteins into the CSF; with the various blood components comes the plasma globulin reflected in the increased LD activity. Control of the infection and diminution in the meningeal inflammation is accompanied by restoration of the meningeal blood-barrier, and the CSF-LD gradually returns to the normal

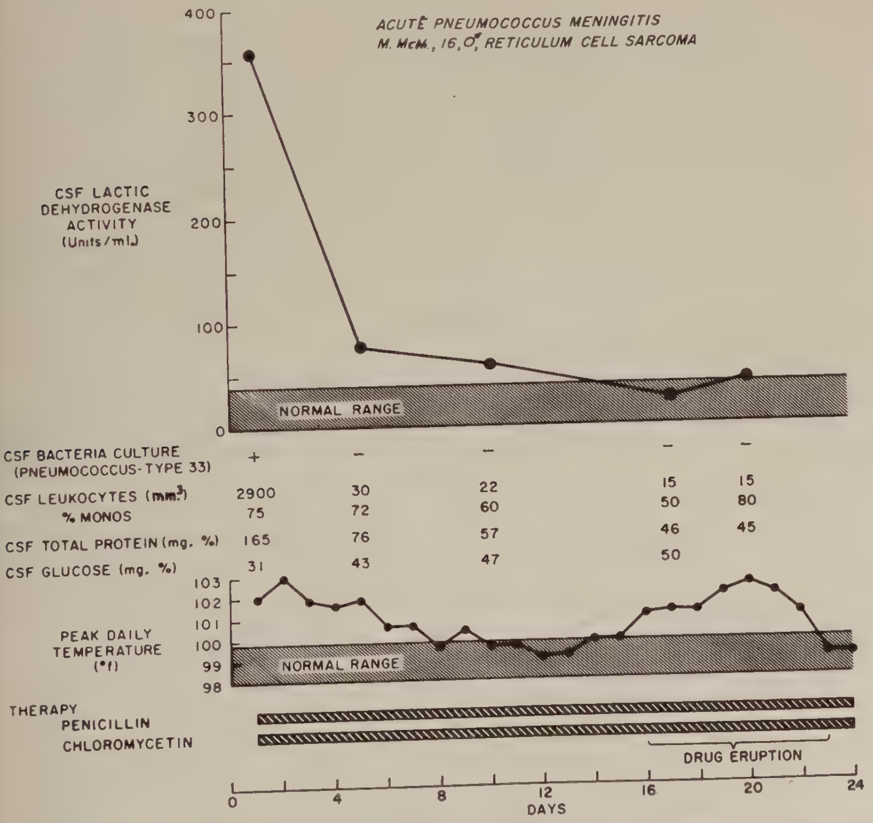

FIGURE 14. Serial lactic dehydrogenase activity and other laboratory parameters of cerebrospinal fluid obtained from a patient with pneumococcus meningitis.

range. Although there may appear to be a relationship between the leukocyte content and the LD activity of the CSF (FIGURE 14), this relationship very probably reflects a common mechanism which explains the parallelism of the two parameters. In some instances of bacterial meningitis, as well as in some patients with leukemic involvement of the central nervous system, the leukocyte content and LD activity of the CSF vary more or less independently. As to the other components of CSF associated with bacterial meningitis—CSF appearance, chloride, glucose, total protein, and serologic reaction—there appears to be no apparent relationship to CSF-LD activity.

Primary and metastatic malignant tissues of the central nervous system may contribute increased LD activity to the CSF. Presumably, the increased CSF-LD activity transpires by a mechanism analogous to that described for the increase of LD activity of media in tissue cultures of malignant cells. The tumor cells within the central nervous system may contribute LD activity to the CSF fluid which bathes the tumor or surrounding tissue; some increased CSF-LD activity may result from the enzyme released from destruction of normal brain tissue. This enzyme contribution will in part depend on the size of the tumor, its rate of growth, and decrease of brain tissue necrosis. With the growth of the malignant tumor, there is a gradual and sustained increase in CSF-LD activity. The CSF-LD activity is independent of the color and the cellular and chemical composition of the CSF (TABLE 8). Therapy that diminishes tumor growth within the central nervous system may result in diminution of CSF-LD activity.

When a tumor of the central nervous system is located in a position that obstructs the flow of CSF from the area bathing or draining the tumor, the increased LD activity may not reach the general reservoir of CSF. This phenomenon is illustrated by the data in TABLE 9, which suggest that, in a case of primary brain tumor, a glioblastoma multiforme of the left temporoparietal lobe obstructed the flow of CSF from the left lateral ventricle, resulting in elevated CSF-LD in the left and normal CSF-LD in the right ventricular fluid. These mechanical factors, as well as others, may account for the finding of normal CSF-LD in some instances of malignant tumors of the central nervous

TABLE 8

MALIGNANT MELANOMA METASTATIC TO LUNGS, BRAIN, LIVER, NODES

Lactic Dehydrogenase Activity and Other Laboratory Data of Cerebrospinal Fluid Obtained from a Patient (M. C., 45 Years, Female) with Malignant Melanoma Metastases to Brain

	Initial pressure	Color	Protein	Glucose	Chloride	CSF-LD
	mm. H_2O		*mg.%*	*mg.%*	*mEq./l.*	*units/ml.*
9/17/57	108	Clear xanthochromic	1430	47	117	400
9/27/57 (expired)	100	Clear xanthochromic	1400	69	107	840

TABLE 9

LEFT PARIETOTEMPORAL LOBE PRIMARY TUMOR GLIOBLASTOMA MULTIFORME

Lactic Dehydrogenase Activity and Other Pertinent Laboratory Data of Ventricular Fluid Obtained from a Patient (9A) with a Primary Brain Tumor that Obstructed the Left Ventricular Fluid Drainage

Patient 9 A

	Lactic dehydrogenase activity	Color	Cells		Total protein
			WBC	RBC	
	units/ml./min.		*per mm.*^{3}	*per mm.*^{3}	*mg.%*
Right lateral ventricular fluid	13	Crystal	0	2,500	15
Left lateral ventricular fluid	380	Yellow	1	20,000	3,600

system. Tumors located extradurally result in no increased CSF-LD activity because the LD produced by the tumor fails to reach the CSF.

Summary

Several different mechanisms appear to explain the alterations in LD activity in serum, serous effusion, and CSF. Similar quantitative changes in LD may result from different mechanisms or combinations of different phenomena. Other factors, including the degradation and metabolic handling of LD, unknown activators, and/or inhibitors, may contribute to the alterations of LD activity in body fluids. An understanding of the known mechanisms contributing to LD activity alteration in body fluids is necessary in order to correlate the quantitative and serial changes in LD activity with the experimental and/or clinical factors.

Acknowledgment

I acknowledge my indebtedness to Philip Cabaud, Barry Decker, Sidney P. Gottfried, Carlos Manso, Alice E. Moore, Kanematsu Sugiura, and Rita Wróblewski for their collaboration, and for their permission to include material as yet unpublished. The technical contributions of Martin Podgainy, Carol Ross, and Joan Pasternak and the secretarial assistance of Carole Laffan cannot be overemphasized.

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SIGNIFICANCE OF SERUM ALDOLASE LEVELS

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Aldolase was probably the first to be discovered of the now long list of tissue enzymes that are normally present in the blood serum and that are found in increased amounts in certain cases of tissue injury. In 1943 Warburg and Christian[1] first demonstrated the presence of five of these enzymes of glycolysis in rat serum and observed that the levels of two of these, aldolase and triose isomerase, were elevated in the serum of tumor-bearing rats. These investigators related the high serum levels to the presence of the malignancy and postulated an increased liberation of the enzyme from the muscles of the animal as a secondary effect of tumor growth. They found no elevation in the blood serum of cancer patients. These observations prompted Lehninger and myself[2, 3] to study further the aldolase activity in the serum of normal and tumor-bearing rats, employing an original assay method that appeared to obviate possible negative errors in certain existing methods and that could be employed in assaying any biological material. Our own observations confirmed those of Warburg and Christian on the rat, but it was felt that the tumor itself was a more probable source of the increased amounts of enzyme in the serum; the evidence supporting this conclusion will be presented. A survey of the aldolase activity in the serum of patients with and without malignant disease was also conducted in the hope that this procedure might be that long-sought desideratum, a blood test for cancer. It was found that some patients with cancer did show significantly elevated aldolase levels (about 20 per cent). However, this was in no way a diagnostic test, and the reason that some cases of malignancy showed an elevation while other apparently similar cases did not was not apparent. It is of interest to note that in early studies high levels were found in two cases of progressive muscular dystrophy and in one case of jaundice.

The problem was approached once more in a series of experimental and clinical studies conducted at the Mayo Clinic, Rochester, Minn.[4-7] The blood sera of more than one thousand patients with a wide variety of pathological conditions were tested. The results of this survey are now well known, as they demonstrated those disease states in which the determination of serum aldolase activity has proved to be of great clinical usefulness. As Bruns and Puls[8] also showed, marked rises occurred in early acute hepatitis, in distinct contrast to the normal or slightly elevated values found in obstructive jaundice and cirrhosis. Subsequent reports[9-14] confirmed these findings and emphasized the fact that the enzyme elevation in the serum did not reflect the status of liver function, but indicated acute cellular damage. Cases of muscular dystrophy and myositis showed elevations while secondary muscular atrophy did not, as had been demonstrated by Schapira *et al.*[15] The value of this test in the differential diagnosis of muscular disease and as a research tool in the study of muscular dystrophy has been demonstrated.[16-20] Isolated instances of high serum aldolase were noted in massive pulmonary infarcts, extensive peripheral gangrene, acute hemorrhagic pancreatitis, and some cases of large malignant tumors. It so happened that no cases of acute myocardial infarction were included in this survey; however, subsequent studies by many investigators have shown that there is a brief but marked rise in serum aldolase activity in this condition.[21, 22]

The serum aldolase did not vary with the sex or age of the individual. Significant changes in level were not seen in extremely debilitated or cachectic patients, in severe anemia, in acute or chronic inflammatory diseases, in endocrine disorders or with hormone therapy, or in postoperative patients.

As evidenced by the recent literature and by the papers presented in this monograph, the clinical usefulness of these enzyme assays is now well established. However, I believe that with present knowledge we should reserve judgment as to which particular enzymes should best be assayed as clinical procedures. Perhaps the estimation of other enzymes, as yet little studied, may prove to be of greater value, and it is likely that comparative studies will show that the particular enzyme determinations of choice will vary with different pathological conditions. The relative changes in serum enzyme levels with disease may vary with the normal content of the enzyme in the tissue and with little-understood factors of cellular permeability to enzymes. For example, Schwarzmann[23] showed that, while both aldolase and the transaminases were markedly elevated in acute hepatitis, the moderate elevations of transaminase in obstructive jaundice, as compared to the slightly elevated or normal aldolase values, appeared to make the latter determination the better clinical test. The relatively large amount of glutamic-oxaloacetic transaminase in cardiac muscle might explain the particular value of this enzyme assay in the diagnosis of myocardial infarction.[24] On the other hand, aldolase is among the enzymes that are in high concentration in skeletal muscle; as a result, its determination might be the more sensitive procedure in studying muscle disease.

There has been relatively little work reported on the basic physiology of serum enzymes. Largely unanswered at this time are the questions: What is the source of the enzymes normally present in serum? What is the source of an abnormal amount of an enzyme in the serum? How are enzymes eliminated from the blood? How is the remarkably normal level maintained? These problems must be solved to appreciate fully the significance of serum enzyme levels. Considering aldolase as representative of this group of enzymes, I shall present some observations, both clinical and experimental, and the conclusions that I believe can be drawn from them.

In a rat with a large tumor (Walker tumor No. 256, transplanted into the back by subcutaneous injection) and hence with a very high serum aldolase level, it was found that the serum level fell rapidly to normal when the tumor mass was surgically removed (FIGURE 1). Experimentally produced conditions similar to those seen in the tumor-bearing animal, such as anemia and cachexia, did not alter the serum aldolase level. The enzyme level was constant, with repeated massive bleeding that produced a fall in hemoglobin concentration to less than 25 per cent of normal (FIGURE 2). Starvation resulting

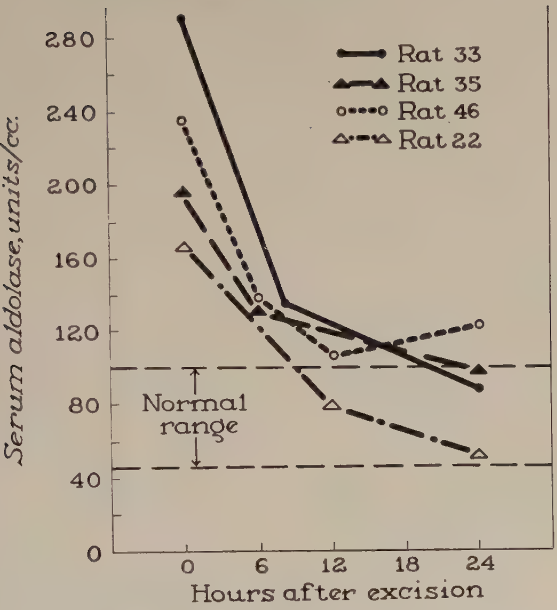

FIGURE 1. Fall in serum aldolse level following excision of a large tumor

in the loss of more than one third of the body weight did not alter the enzyme level (FIGURE 3). It was found that the enzyme content of blood from a vein leaving the tumor was always significantly higher than that of arterial blood drawn at the same time (FIGURE 4). There was no significant change in the aldolase content of the various tissues of a tumor-bearing animal as compared to normal tissue values. Sham operations or the presence of severe infection did not influence the aldolase level. It thus seemed apparent that the high serum enzyme level in the tumor-bearing rat was due to the presence of the tumor itself, not to a secondary effect of the malignancy upon another tissue, and that an excess of the enzyme was actually being liberated by the tumor mass.

A possible explanation for this excessive liberation came from the survey of the serum aldolase levels of patients with a variety of diseases. Only a small percentage of cases of cancer showed high levels, and often massive tumors had normal values; certainly the presence of malignant tissue per se did not cause an elevation of serum aldolase. However, high serum values were always noted in conditions with extensive, acute cellular damage, such as acute hepatitis, pancreatitis, and pulmonary infarction. Returning to the animal studies, it was noted that sections of the Walker tumor always showed many small areas of necrosis. Likewise, liver necrosis in the rat, as produced experimentally by the inhalation of carbon tetrachloride vapors or the intravenous injection of papain, resulted in an immediate marked rise in the serum aldolase level that promptly returned to normal with healing. It was noted that the rate of fall to normal approached that following extirpation of a large tumor

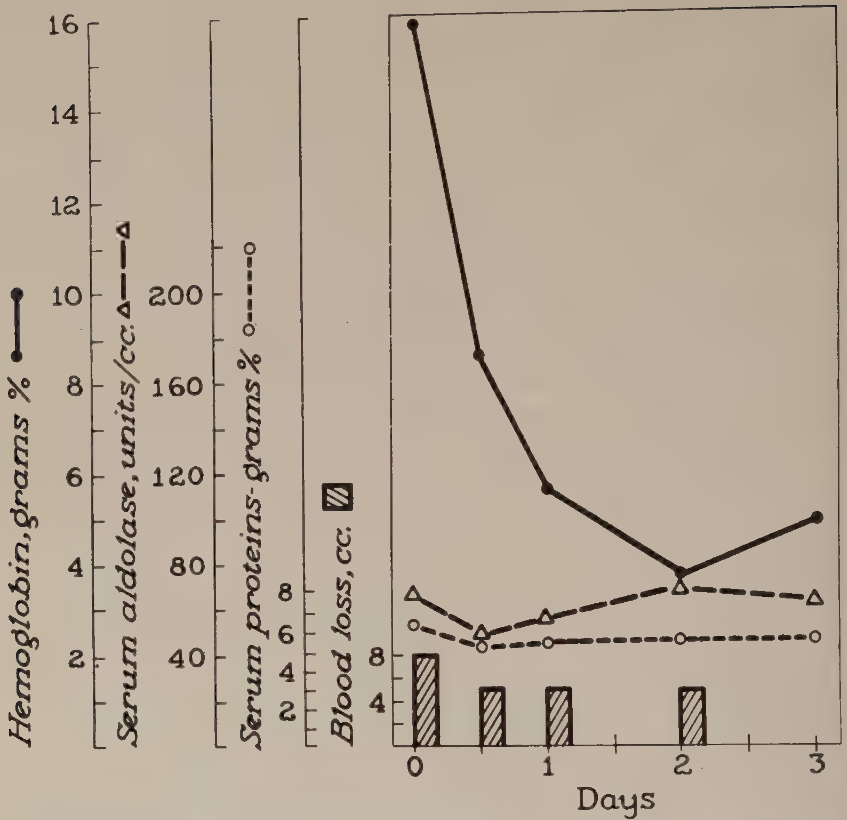

FIGURE 2. Effect of repeated massive blood loss in the rat on serum aldolase, total serum protein, and blood hemoglobin concentration.

or following intravenous injection of crystalline aldolase (FIGURE 5), suggesting a sudden, brief liberation from the damaged liver tissue.

As suggested by Warburg and Hiepler[25] and by Schade[26] in their studies of aldolase liberation from the cells of the mouse ascites tumor, we tested the enzyme liberation from tissue slices. The liberation was slow in a physiological solution with available glucose and oxygen, but without glucose, or under anaerobic conditions, there was a marked increase in the aldolase content of the suspending medium (FIGURE 6). It was felt that these experiments demonstrated that conditions detrimental to the life of the cell, or at least to the integrity of the cellular membrane, resulted in a release of the enzyme from the cells. It was interesting to note that, when the experiment was performed using a slice of liver, a lack of oxygen resulted in the greater liberation of aldolase while, in the case of a slice of tumor tissue, lack of glucose had a more pronounced effect than did anoxia. This would accord with the known greater importance of glycolysis than respiration in tumor cell metabolism as compared to that of most normal adult tissues. A similar enzyme liberation from excised rat diaphragm was shown in the studies of Zierler,[27] who demonstrated that within certain time limits the effect was reversible, thus demonstrating that actual cellular death was not necessary for the liberation of enzyme.

These studies would indicate that an abnormally high serum aldolase content

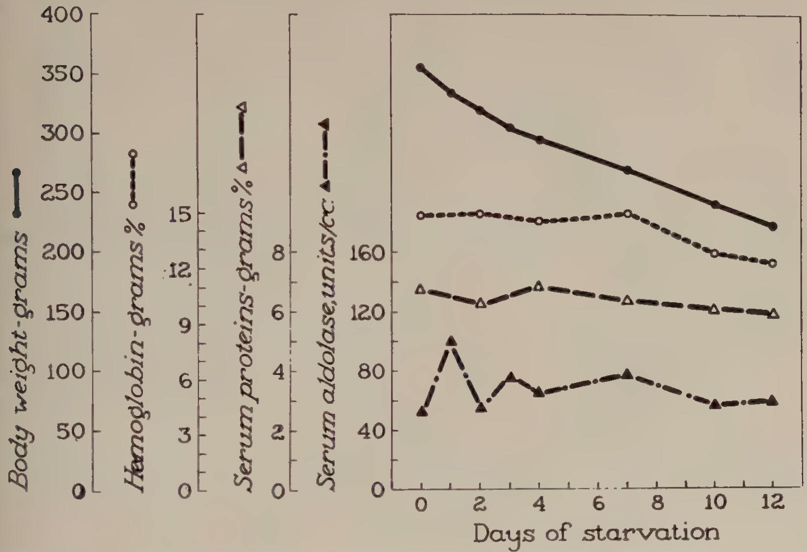

FIGURE 3. Effect of starvation in the rat on serum aldolase, body weight, total serum protein, and blood hemoglobin concentration.

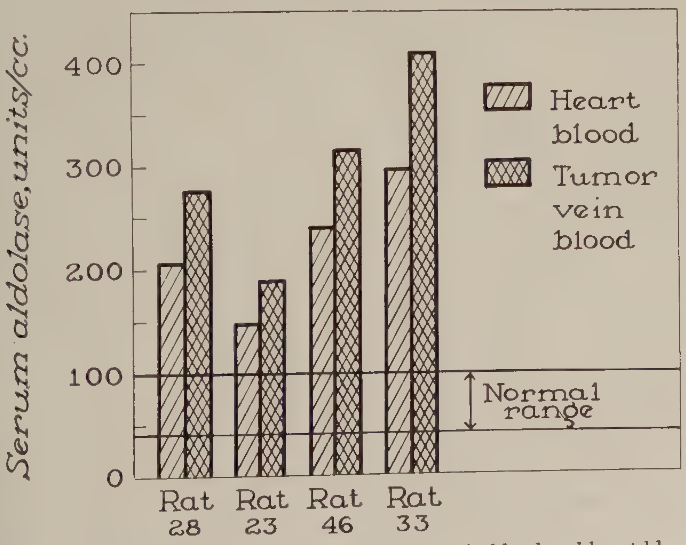

FIGURE 4. Comparison of aldolase content of tumor-vein blood and heart blood drawn at the same time in tumor-bearing rats.

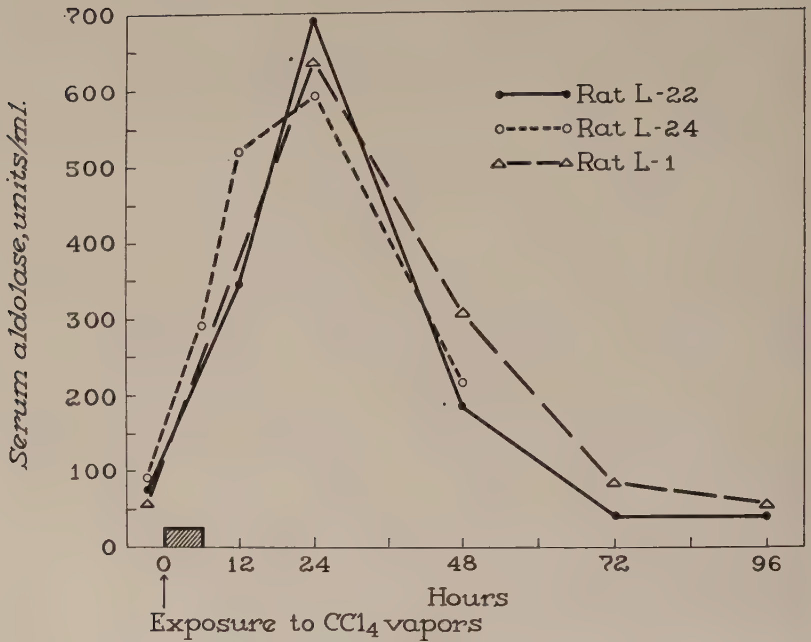

FIGURE 5. Changes in serum aldolase in the rat due to experimental hepatic necrosis

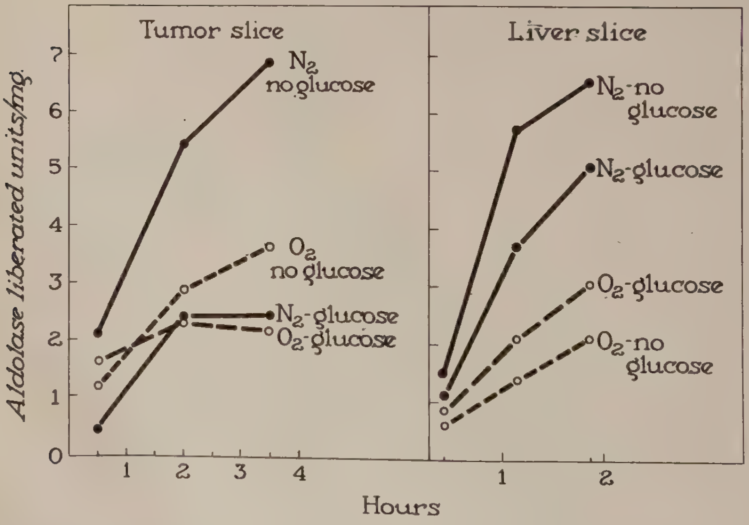

FIGURE 6. Rate of liberation of aldolase into the suspending medium from tissue slices under varying conditions.

results from the rather sudden injury of many cells. It is probably more exact to speak of tissue injury, which would include relatively mild and sometimes reversible changes in the cell membrane, rather than to use the term necrosis, which implies a destruction of the cells demonstrable by histological examination. This concept would explain those instances of elevation in serum enzyme values without finding gross or microscopic evidence of necrosis of some tissue. The normal enzyme content might be the result of intracellular enzymes freed in the normal constant turnover of body cells; alternatively, there may be a slight leakage from healthy cells, a possibility suggested by Zierler's experiments on enzyme liberation from intact muscle tissue.

A physiological mechanism must exist to maintain the normal level of a serum enzyme activity at such a remarkably constant value, probably representing a dynamic equilibrium between constant production and elimination. It was noted that if the source of the excess enzyme be removed, as by excision of a large tumor, the level fell rapidly to normal. Similarly, the level returned promptly to normal after the rise resulting from acute liver necrosis. If crystalline aldolase is injected intravenously, there is an immediate rise relative to the amount injected and the blood volume of the animal, followed by a steady fall to normal at a constant rate (FIGURE 7). In all cases the rate of fall was about the same.

As to the possible means of elimination of aldolase from the blood, in the rat it was found that following bilateral nephrectomy there was a steady rise in the serum enzyme level while the animal lived (FIGURE 8). The same rise occurred after bilateral ureteral ligation, ruling out a nonexcretory renal function. However, when crystalline aldolase was injected intravenously into a nephrectomized rat the immediate high level fell, but at a slower rate than when normal renal function was present (FIGURE 9). In the rat it thus appeared that renal excretion might be a factor in the elimination of the serum enzyme, but was not the only mechanism operating. I was unable to detect aldolase activity in the urine of either normal animals or in those with a very high serum level; however, the enzyme may have been present in an inactive form. A species difference may be present, nevertheless, as clinical studies showed that severe renal insufficiency, even terminal uremia, did not result in elevated serum levels.

In the rat, ligation of the bile duct, although it produced severe jaundice and led to the death of the animal, did not elevate the serum aldolase level. This is consistent with the finding of normal values in patients with obstructive jaundice. Excretion in the bile thus is not important in the elimination of the enzyme. The severe physiological derangement after total hepatectomy in the rat made such experiments difficult to interpret. However, in patients severe hepatic insufficiency, even terminal hepatic coma, was associated with normal serum aldolase levels. The attempted blocking of the reticuloendothelial system by the intravenous injection of India ink did not elevate the normal serum enzyme level, nor did it delay the fall in level after the intravenous injection of crystalline aldolase. However, a complete supression of phagocytosis by this means may not be possible.

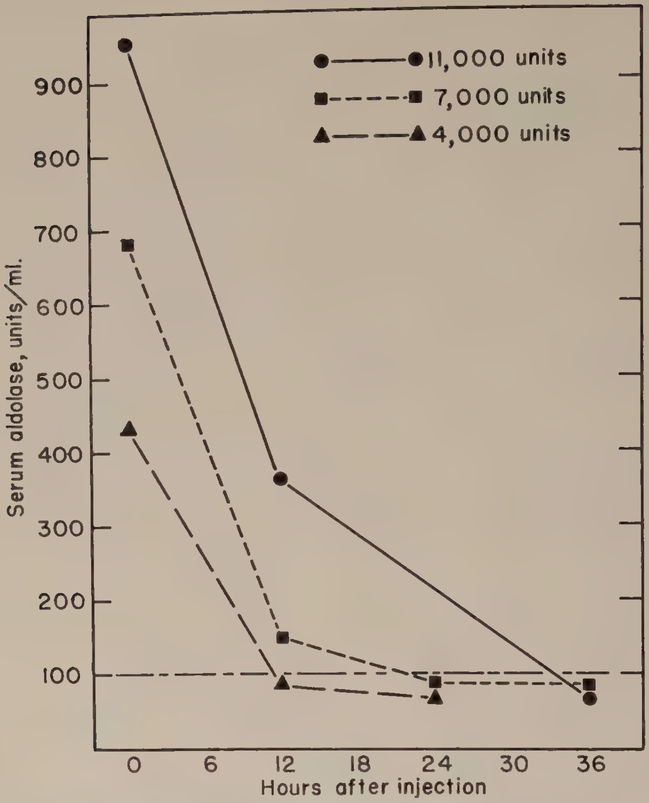

FIGURE 7. Serum aldolase levels following the intravenous injection of known amounts of crystalline aldolase.

The mode of elimination of this enzyme remains undetermined, although these experiments suggest that renal excretion plays a role. It is hoped that further studies on aldolase and other serum enzymes will throw more light on this problem. Before the full clinical significance of serum enzyme values may be appreciated, much more must be known about the physiological processes that control these levels.

Summary

The discovery in 1943 of aldolase activity in blood serum initiated this phase of serum enzymology, as this tissue enzyme was probably the first shown to be present in the serum in increased amounts as a result of tissue injury and the first whose level proved to be of clinical significance.

Both clinical and experimental evidence is presented to show that damage to a tissue rich in the enzyme will result in a liberation of aldolase into the blood. Although in most instances this cellular damage represents frank necrosis, liberation may occur without histological evidence of cellular destruction.

The physiological mechanism controlling the level of an enzyme in the blood

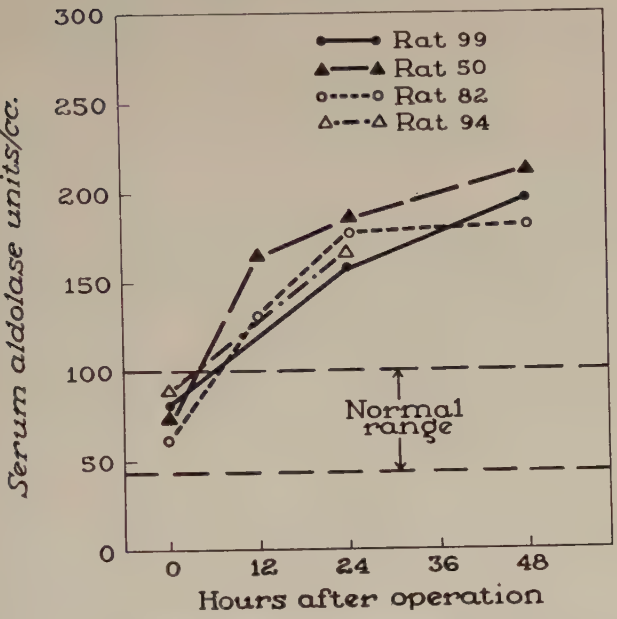

FIGURE 8. Serum aldolase levels in the rat following bilateral nephrectomy

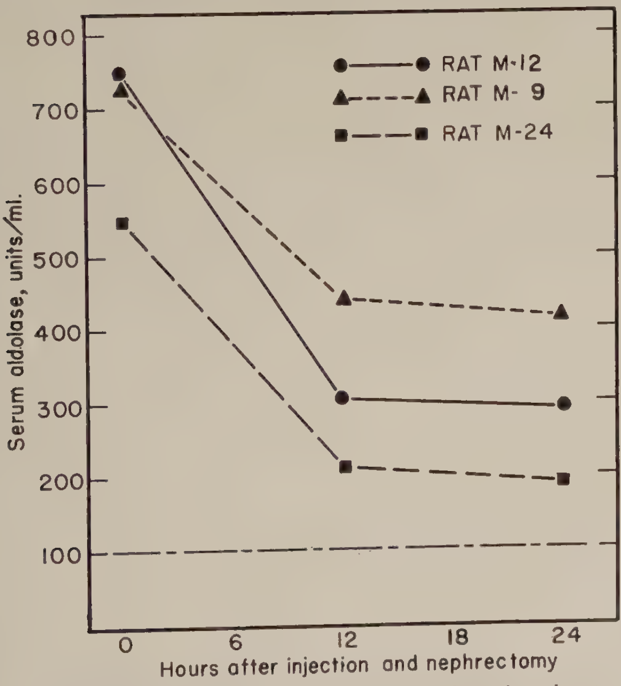

FIGURE 9. Serum aldolase levels in the rat following bilateral nephrectomy and simultaneous intravenous injection of crystalline aldolase.

serum and the means of elimination of the enzyme from the blood has not been determined, but it is of great importance in understanding the significance of the serum enzyme level. Experiments relative to this question are presented. It is suggested that, in the rat, renal excretion may be one, but not the only, factor in the elimination of aldolase.

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Discussion of the Paper

H. J. Leppelmann (*University of Münster, Münster, Germany*): The findings of Sibley with reference to serum aldolase do not agree at all points with our own. I believe that there are two reasons for these differences: first, the relatively small increase in serum aldolase as compared with those of GOT and LDH; second, the essential dependence of the increase in aldolase upon the severity of the disease.

SOME ENIGMAS IN THE COMPARISON OF MULTIPLE SERUM ENZYME LEVELS*

By Laurens P. White

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It is generally assumed that changes in the serum levels of certain enzymes reflect damage to tissues that contain these enzymes. The damage may be from necrosis, whether ischemic, infectious, or traumatic, and it results in the outpouring of the enzymes contained within the damaged cells. The kinds of damage that produce these changes have not been thoroughly explored, nor has the role of changes in cell membrane permeability.

Despite these deficiencies of factual knowledge, one might suppose that a pattern of serum enzyme abnormalities would bear some direct relationship to the enzyme content of a specific injured tissue and that, after study of the pattern, it would permit certain inferences as to which tissue was damaged.

The ubiquity of most glycolytic enzymes might, however, lead to a patternless abnormality, regardless of the site of tissue damage.

In the course of studies in the mechanism(s) responsible for serum enzyme abnormalities in patients with disseminated cancer, it became apparent that there were very definite patterns of serum enzyme abnormalities for several diseases: specifically, cancer, myocardial infarction, infectious hepatitis, and muscular dystrophy. A description of these patterns and some remarks on the pattern in cancer patients form the basis of this report.

Patients. Six hundred and fifty patients with a variety of diseases were the source of the blood for these studies; patients with cancer, myocardial infarction, hepatitis, and muscular dystrophy have been followed with serial enzyme assays for periods as long as 18 months.

Methods. The enzymes measured were: aldolase,[1] phosphohexose isomerase,[2] lactic dehydrogenase[3] (LDH), glutamic-oxaloacetic transaminase (SGOT),[4] isocitric dehydrogenase (ICD)[5] and, in a few patients, alcohol dehydrogenase. The assays were performed in a standard manner according to methods described elsewhere.[6-8]

Blood samples were taken with extreme care to avoid hemolysis, and were run within 24 hours. Tissue samples from mice and rabbits were homogenized in a Potter-Elvehjem type apparatus in water, as described by Meister,[9] and analyzed immediately for LDH and ICD content. The protein content of the homogenate was measured by the method of Lowry *et al.*[10]

Results. TABLE 1 shows the results of multiple determinations in 17 patients with disseminated cancer. Four of these patients exhibited clinical and laboratory evidence of liver metastases and revealed abnormal serum values for LDH, aldolase, isocitric dehydrogenase, and hexose isomerase. Thirteen patients without evidence of liver metastases showed a different pattern, with no ele-

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TABLE 1
SERUM ENZYME LEVELS IN PATIENTS WITH METASTATIC CANCER

	LDH	Aldolase	ICD
Patients without liver metastases			
	μ/0.01 ml.	*μ/ml.*	*mμM/ml.*
Hall	350	14.6	193
Gust	132	4.6	116
Beat	128	6.5	127
Roge	151	3.6	165
Dunn	148	8.8	234
Dash	648	25.8	236
Cart	173	17.6	176
Gayl	158	9.9	240
Mart	138	10.7	192
Rice	253	18.6	182
Lund	787	8.9	236
Maye	377	6.4	132
Whit	123		275
Patients with liver metastases			
Bull	179	14.5	825
Jiji	150	10.6	550
Conl	307	19.7	297
Garn	172	11.5	774
Normal	<110	<9.5	<270

TABLE 2
SERUM ENZYME LEVELS IN PATIENTS WITH MUSCULAR DYSTROPHY

Patient	LDH	Aldolase	Isomerase	ICD
	μ/0.01 ml.	*μ/ml.*	*μ/0.04 ml.*	*mμM/ml.*
Waeg	371	70.0	25	132
Rice	212	28.5	25	126
Russ	354	78.0	25	108
Doug	417	28.8	30	144
Gard	526	112.0	67	228
Frit	588	79.0	84	208
Bfri	658	108.0	128	288
Prue	255	35.0	22	318
Normal	<110	<9.5	<40	<270

vated ICD values and no normal LDH. Aldolase levels were abnormal in 6 of 12 patients and hexose isomerase in 5 of 13. In a group previously described,[7] 19 of 21 patients who had abnormal serum levels of LDH and aldolase excreted abnormal amounts of creatine in their urine. In the present group, 24-hour urine creatine determinations were performed in 5 patients and revealed creatinuria in 4 of the 5.

TABLE 2 shows the enzyme levels of 8 patients with muscular dystrophy of he progressive type described by Duchenne showed a pattern similar to that f patients with cancer. This included abnormal LDH values, tremendously levated levels of aldolase, hexose isomerase normal in 5 of the 8, and ICD hat was normal in 6 and barely elevated in 2. (The actual normal level for CD in children under 10 years may well be higher than that in adults, and hese two values may, in fact, be normal.) These results are in agreement with those of Dreyfus and Schapira.[11] The pattern of serum enzymes in patients with muscular dystrophy reveals a marked increase in aldolase and, to a lesser extent, in LDH, an occasional elevation in hexose isomerase; and, probably, normal ICD levels.

TABLE 3 shows the levels of 13 patients with recent myocardial infarction studied with LDH and ICD assays. Each patient had an elevated serum LDH value; only one had an abnormal ICD level. This patient was in profound congestive failure, with marked hepatomegaly, and had a minimal elevation of ICD level. The pattern in patients with myocardial infarction is elevation of LDH (and of SGOT, isomerase, and aldolase), with normal ICD level.

TABLE 4 shows the levels of 9 patients with infectious hepatitis who had simultaneous LDH and ICD assays. In each case the ICD value was markedly elevated, while LDH was only moderately increased or, in two cases, at normal levels. Two of these patients had elevated serum levels of glutamic-oxaloacetic transaminase. The pattern is one of marked elevation of ICD and minimal elevation of LDH.

A comparison of the levels of LDH and ICD in animal tissues is of importance in attempting to understand these patterns.

As shown in TABLE 5 levels of LDH in skeletal muscle are high, with lower values in heart muscle, liver, and tumor. ICD values, however, show equal amounts in heart muscle and in liver, with much lower levels in skeletal muscle and in tumor. These values in mouse tissues are comparable to those

TABLE 3

SERUM ENZYME LEVELS FOLLOWING MYOCARDIAL INFARCTION

Patient	LDH	ICD
	µ/0.01 ml.	*mµM/ml.*
Dani	178	165
Hami	428	162
Hoop	710	306
Olso	163	144
Smit	211	94
Ayer	140	171
Parr	470	192
MacL	391	186
Zoll	201	288
Edwa	410	180
Patt	248	193
Stil	241	222
Grac	184	189
Average	306	192
Normal range	63 to 103	60 to 270

TABLE 4

SERUM ENZYME LEVELS IN PATIENTS WITH INFECTIOUS HEPATITIS

Patients	LDH	ICD
	µ/0.01 ml.	*mµM/ml.*
Ladb	133	825
Sena	253	2750
West	163	1650
Woos	153	1397
Mark	96	715
Niel	130	605
Rike	123	440
Geor	95	550
Nich	158	1512
Average	145	1160
Normal	<110	<270

TABLE 5

LDH AND ICD IN VARIOUS MOUSE TISSUES

Tissue	LDH	ICD
	µm/min./mg. Protein 38° C.	*mµM/min./mg. Protein 25° C.*
Heart	19.2 to 24.7	115 to 125
Skeletal muscle	36.5 to 50.0	18 to 23
Liver	8.2 to 17.9	128 to 150
Tumor	13.5 to 21.9	47 to 49

described by Meister,[9] and by Adler and Von Eüler;[12] similar values have been obtained in rabbit tissue.

On reviewing the patterns of serum enzyme abnormality with these data in mind, one is struck by a curious anomaly; namely, the surprising fact that patients with myocardial infarction involving, presumably, only a few grams of muscle should have high serum LDH, while patients with hepatitis, involving the entire liver, should have only a moderate deviation of LDH. By the same comparison, the ICD abnormalities are more intelligible, since a large amount of tissue damage may be required to elevate the ICD levels, and myocardial infarction does not involve this critical amount of tissue. One cannot have it both ways, however, and if a myocardial infarction does not involve enough tissue to raise the ICD level in serum, LDH values should also be normal, unless they are subject to different fates in serum or leak out of cells at different rates. Since neither enzyme is appreciably affected by incubation with albumin or with serum, one is inclined to study the permeability of cell membrane to specific enzymes in the hope of explaining the discrepancy.

LDH and ICD are present in appreciable amounts in mouse tumors. For LDH this is considerably less than the concentration in skeletal muscle, while for ICD the amount is greater than for such muscle. If the source of abnormal amounts of LDH (and of aldolase) in the serum of animals with cancer were the tumor, then by necrosis, which is so frequent, abnormal serum levels of ICD should also be found, assuming equal rates of liberation. This has not been the case, except in patients with liver metastases (this supposition has the same limitations as those relative to liberation of LDH and ICD from liver in hepatitis). It is tempting to speculate, as did Warburg,[13] that tumor is not the source of abnormal amounts of aldolase and LDH in serum, or perhaps not the only source.

Again referring to the patterns of serum enzyme abnormality, it is apparent that the pattern in cancer patients who do not have demonstrable liver metastases resembles that in myocardial infarction and muscular dystrophy. Furthermore, almost all cancer patients who have abnormal serum levels of aldolase, LDH, and phosphohexose isomerase also excrete abnormal amounts of creatine in their urine, as do patients with muscular disease. In some patients it seemed possible that muscle wasting, which so frequently accompanies the growth of a malignant tumor, might be responsible both for the creatinuria and the serum enzyme abnormalities.

It must be pointed out that simple starvation is not associated with abnormal levels of aldolase lactic dehydrogenase or of isomerase. The observations of Mider and his co-workers have shown, however, that the starvation is not at all simple in cancerous rats.[14] In a series of elegant experiments they were able to demonstrate that a large proportion of the protein in tumor could have been derived only from muscle, and that the deficit of muscle protein was very close to the difference between the total nitrogen content of tumor and the net nitrogen balance of the animal. The marked muscle wasting could be prevented, to some extent, by force-feeding the anorectic, tumor-bearing animals. The observations support Mider's concept of tumor as a nitrogen trap, and also the experiments of Zamecnik[15] and of LePage *et al.*[16] demonstrating the tenacious retention by tumors of their protein, even in the face of severe starvation.

If the creatinuria and serum enzyme abnormalities in cancer patients were due to muscle wasting, secondary to a diet inadequate to supply the needs of both the tumor and the nontumorous body, it should be possible to reverse the abnormality by the administration of very large amounts of food, chiefly protein (such feeding should have no effect on tumor necrosis).

Such feeding experiments have been performed in 21 patients, described more completely elsewhere.[7] One of the most striking of these is shown in FIGURE 1.

In other cancer patients similar changes in serum enzyme levels have followed the intravenous administration of protein hydrolyzate (Amigen), or of human serum albumin and, in one case, the oral intake of protein at a level of 100 gm./day. In none of these patients was there a change in hematocrit following the intravenous infusion, nor was there sudden change in weight. In 10 patients, however, similar treatment failed to change the serum enzyme concentrations. All such patients had evident liver metastases.

Similar intravenous administration of Amigen in 5 patients with muscular dystrophy, all with creatinuria and elevated serum level of LDH, aldolase, and hexose isomerase did not lead to changes in any of the enzymes measured.

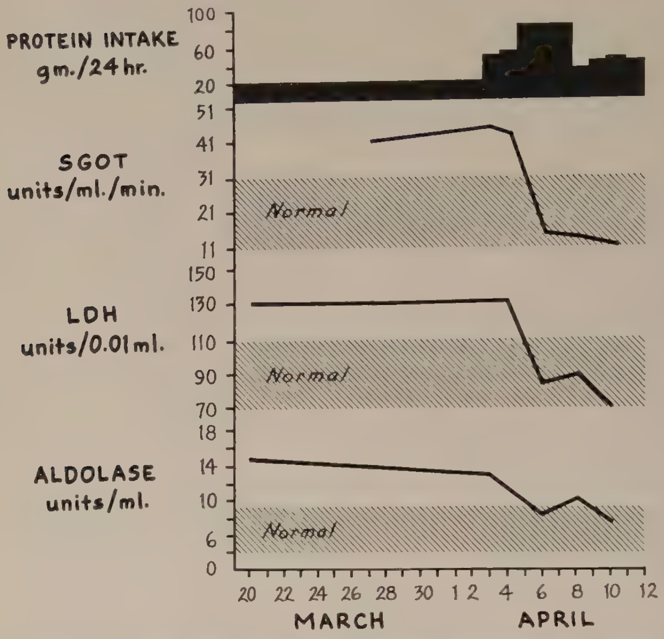

FIGURE 1. A 54-year-old male patient (Mont.) with carcinoma of the floor of the mouth and supraclavicular metasteses. Protein intake averaged 20 gm./day during the control period. There was a rapid fall in all serum enzymes measured following the intravenous injection of Amigen in amounts up to 75 gm./day. Coincidentally, tumor growth was markedly accelerated.

Discussion

The measurement of enzymes in blood serum affords, at any time, a static picture of what must be a dynamic situation; various sources may contribute to the enzyme content of the serum. Normally, these appear to be in equilibrium with the site(s) of excretion or inactivation. Warburg[13] has shown, as has Sibley,[1] that a great reserve for elimination of excess amounts of aldolase exists. Elevation of serum levels of various enzymes must therefore follow only when this ability of the body to eliminate the enzymes is exceeded by the rate at which they enter serum. These rates, both into and out of blood, may vary for each enzyme. I have some data, as yet unpublished, indicating that the rates of elimination of aldolase and of LDH are similar.

This argument does not explain the failure of LDH to rise to very high levels in hepatitis or in muscular dystrophy or of ICD to increase following myocardial infarction. These failures, however, result in certain patterns of enzyme levels that are distinctive for particular diseases. Until more is known of factors related to change in the permeability of cells to their enzyme content and of the excretion of enzymes, these patterns will remain enigmas.

The pattern in cancer patients without liver involvement is similar to that of patients with muscle diseases, as is their excretion of creatine. These two phenomena could be unrelated, although simultaneous. Muscle wasting from starvation is not associated with serum enzyme abnormalities, perhaps because the rates of wasting and of possible pouring of enzymes into blood are not sufficiently great. In cancer this rate may be augmented by the added, constant, and increasing drain of the imperious, miserly tumor. The tumor, therefore, may increase the rate of muscle breakdown beyond the ability of the body to secrete the cellular products, with resulting rise in serum aldolase, LDH, and hexose isomerase. The observation that large amounts of protein have apparently reversed the enzyme abnormality supports this thesis as to the origin of the enzyme excess. Sibley,[1] Hill,[3] and Bodansky[2] have all concluded that abnormal serum levels of aldolase, LDH, and hexose isomerase were a result of necrosis within tumor, with outpouring of the contents of necrotic tumor cells. Studies in the arteriovenous differences in blood entering and leaving tumor have not been conclusive. Certainly, necrosis of tumor must contribute in some measure to the enzyme content of blood; it does not explain creatinuria. One can imagine a situation in which tumor necrosis contributed a certain percentage of the excess enzyme, and muscle wasting the remainder. This has been observed in a patient in whom the intravenous administration of human serum albumin was followed by an abrupt fall of aldolase and hexose isomerase, but not to normal values.

Conclusion

Among the mechanisms for producing abnormal serum values of several enzymes one may include: necrosis of tissue (such as muscle, liver, or tumor), inflammation of tissue with or without necrosis, and a distant effect of malignant tumors on body tissues in general, and on muscle in particular, due to the breakdown of these normal tissues to supply the protein for the growth of cancer. Valid explanations of these mechanisms can be reached only indirectly by a study of serum enzymes and must be explored at the level of the cell.

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SERUM 5-NUCLEOTIDASE: CHARACTERIZATION AND EVALUATION IN DISEASE STATES*

By Irving I. Young

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In addition to the nonspecific alkaline and acid phosphatases, mammalian tissues and serum contain another phosphatase, designated as 5-nucleotidase.[1, 2] The 5-nucleotidase of bull semen has been purified and well characterized by Heppel and Hilmoe,[3] and the enzyme present in human serum appears to be similar to it in many respects.[4] Serum 5-nucleotidase can be distinguished from the nonspecific phosphatases by a number of characteristics, including:

(1) *Substrate specificity.* This enzyme specifically hydrolyzes nucleotides in which the phosphate is attached to the 5 position of the pentose, such as adenosine-5-phosphate and inosine-5-phosphate. Adenosine-3-phosphate and other phosphate esters, including β-glycerophosphate and phenyl-phosphate, are unaffected.

(2) *Optimum pH.* Serum 5-nucleotidase is an alkaline phosphatase with a *p*H optimum between 7.5 and 8.0.

(3) *Effect of magnesium ion.* Addition of magnesium ion to the substrate in optimum concentration results in a marked activation of 5-nucleotidase, averaging a threefold increase in activity.

(4) *Effect of ethylenediaminetetraacetic acid (EDTA).* Serum 5-nucleotidase has a greater resistance to inactivation by EDTA than does the nonspecific alkaline phosphatase.

(5) *Electrophoretic mobility.* The activity of 5-nucleotidase is consistently localized to the β-globulin fraction of both normal and pathological human sera, whereas both the nonspecific acid and alkaline phosphatases are α-2-globulins.

Assay of 5-nucleotidase in serum by determining the rate of hydrolysis of a 5-nucleotide such as adenosine-5-phosphate is complicated by the fact that the nonspecific alkaline phosphatase of serum also hydrolyzes this substrate. This has been circumvented by preliminary incubation of serum with 0.0015 M ethylenediaminetetraacetic acid, which inactivates the alkaline phosphatase, but not the 5-nucleotidase. The rate of hydrolysis of adenosine-5-phosphate has then been determined at *p*H 7.5 in the presence of 0.04 M magnesium ion, and the unit of 5-nucleotidase activity has been designated as equivalent to the liberation of 1 mg. of phosphate ion in 1 hour; this unit is analogous to the Bodansky unit of phosphatase activity.[4]

In a group of 30 normal adults serum 5-nucleotidase activity has ranged from 0.3 to 3.2 units per 100 ml. of serum. Values do not appear to be influenced by age, sex, or race, and are fairly constant from week to week in the individual.

Significant elevation of serum 5-nucleotidase activity has been observed thus far only in the presence of hepatobiliary disease. Normal 5-nucleotidase values

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have been found in a variety of commonly observed infectious, neoplastic, and metabolic disorders not involving the liver or biliary tract. In striking contrast to the nonspecific serum alkaline phosphatase, patients with diseases of bone associated with increased osteoblastic activity consistently have normal 5-nucleotidase values. This has been true of subjects with metastatic carcinoma to bone, Paget's disease, and osteomalacia secondary to sprue.

Since significant elevations of serum 5-nucleotidase are apparently observed only in hepatobiliary disease, a study of 5-nucleotidase levels has been undertaken in a group of patients with jaundice of varied etiology, and comparison has been made with simultaneously determined serum alkaline phosphatase values. This series of 127 patients included 39 with extrahepatic biliary tract obstruction, 13 with malignant obstruction, and 26 with common duct stone and other benign causes. There were 88 cases of diffuse hepatocellular damage, including 26 with acute viral hepatitis and 62 with portal and postnecrotic cirrhosis.

The distribution of initial serum alkaline phosphatase values expressed as Bodansky units (B. U.) is summarized in TABLE 1. In approximate agreement with many previous studies,[5] 62 per cent of patients with biliary obstruction had initial values over 15 B. U., and only a rare case fell within the normal range. Only 1 of 88 subjects with hepatocellular damage had an initial value over 15 B. U., but a significant number (26 per cent) fell into the moderately elevated group.

As noted in TABLE 2, a marked elevation of serum 5-nucleotidase has arbitrarily been set as greater than 18 units, and such levels were observed in 67

TABLE I

SERUM ALKALINE PHOSPHATASE VALUES IN 127 CASES OF JAUNDICE

	No. of Cases	Serum alkaline phosphatase			
		Normal Less than 5	Slight rise 5 to 9	Moderate 9 to 15	Marked Greater than 15
Extrahepatic obstructive jaundice	39	3%	5	30	62
Hepatogenous jaundice	88	29%	44	26	1

TABLE 2

DISTRIBUTION OF SERUM 5-NUCLEOTIDASE VALUES IN OBSTRUCTIVE AND HEPATOCELLULAR JAUNDICE

	No. of Cases	Serum 5-nucleotidase			
		Normal Less than 3	Slight rise 3 to 10	Moderate 10 to 18	Marked Greater than 18
Extrahepatic obstructive jaundice	39	0%	5	28	67
Hepatogenous jaundice	88	31%	59	10	0

per cent of patients with biliary tract obstruction; the highest recorded value was 150 units. A normal initial level was not found in this series, although more recently a case of common hepatic duct carcinoma has been observed with a high initial alkaline phosphatase and normal 5-nucleotidase. Two cases of biliary tract obstruction were noted with values less than 10 units. In both, hepatocellular damage was superimposed on the extrahepatic obstruction. In one, an unexplained acute diffuse necrosis was associated with an ampullary carcinoma; in the other, severe portal cirrhosis was associated with common duct stone. Subjects with hepatocellular jaundice usually have initial values under 10 units (90 per cent); in this series none had levels over 18 units. It is evident that the over-all distribution of values in patients with hepatobiliary disease is fairly similar for the two enzymes, although the overlap between obstructive and hepatocellular disease is clearly less with the 5-nucleotidase determination (FIGURE 1).

The serum enzyme levels tend to parallel each other, but do not necessarily do so, and, occasionally, striking discrepancies are observed. Both alkaline phosphatase and 5-nucleotidase levels often fluctuate considerably in subjects with biliary tract obstruction due to common duct stone. It has been observed that, with relief of obstruction, the 5-nucleotidase appears to fall to normal somewhat more rapidly than the alkaline phosphatase and therefore tends to have less significance in the face of rapidly decreasing jaundice.

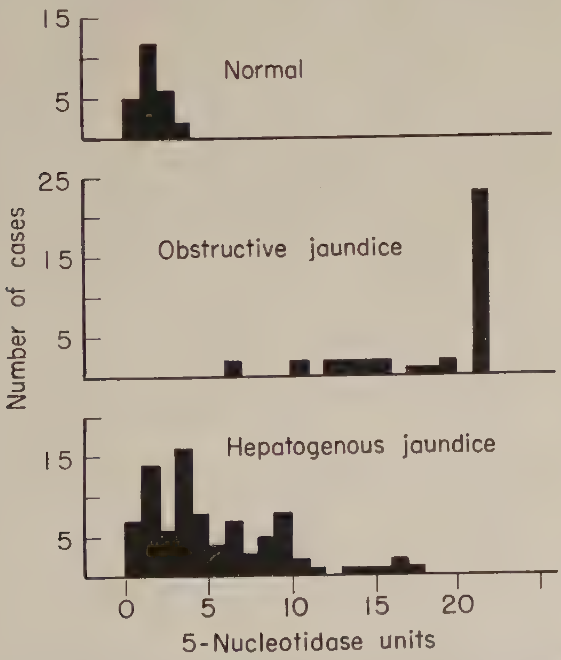

FIGURE 1. Distribution of initial 5-nucleotidase values in normal subjects and patients with obstructive and hepatocellular jaundice. Values over 20 units have been placed in a single column and range up to 150 units.

While 5-nucleotidase levels are usually under 10 units early in the course of acute hepatitis, occasional subjects will have elevations into the obstructive range either at the height of the icterus or shortly thereafter. This usually parallels but occasionally exceeds the rise in serum alkaline phosphatase. In any individual case this elevation can usually be distinguished from the high value in biliary tract obstruction by simultaneous determinations of the serum transaminases that are characteristically high, but fall rapidly as the 5-nucleotidase level rises (FIGURE 2).

The association of increasing jaundice with a low or decreasing serum alkaline phosphatase is characteristic of severe hepatocellular injury. This is even more striking and consistent in the case of serum 5-nucleotidase. In a series of 13 cases of advanced hepatocellular injury with hepatic coma, the serum 5-nucleotidase was observed to be within the normal range in all cases, whereas simultaneously determined alkaline phosphatase values varied from 2.7 to 10.2 B. U. (TABLE 3).

Infiltrative lesions of the liver such as granulomas and neoplasms also result in elevations of serum 5-nucleotidase. On the other hand, infiltrative lesions of bone do not affect 5-nucleotidase levels. FIGURE 3 illustrates the observation that the high alkaline phosphatase due to metastatic carcinoma of bone

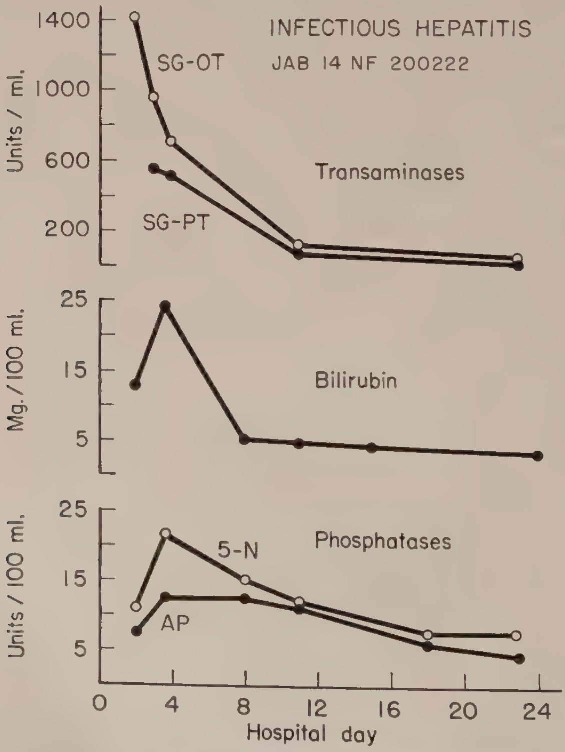

FIGURE 2. Serum phosphatases and transaminases in a subject with acute viral hepatitis.

TABLE 3

SERUM PHOSPHATASE VALUES IN ADVANCED LIVER DISEASE WITH COMA

	5-Nucleotidase	Alkaline phosphatase
Cirrhosis		
W. C.	0.7	6.2
M. F.	1.0	5.4
O. S.	1.2	6.3
S. K.	2.7	3.8
R. C.	0.8	10.2
J. O.	1.7	5.1
R. B.	1.0	2.9
W. G.	2.6	9.5
A. W.	3.1	2.7
R. S.	1.0	3.5
R. C.	0.9	5.2
L. P.	1.3	8.7
Hepatitis		
B. C.	1.8	6.8

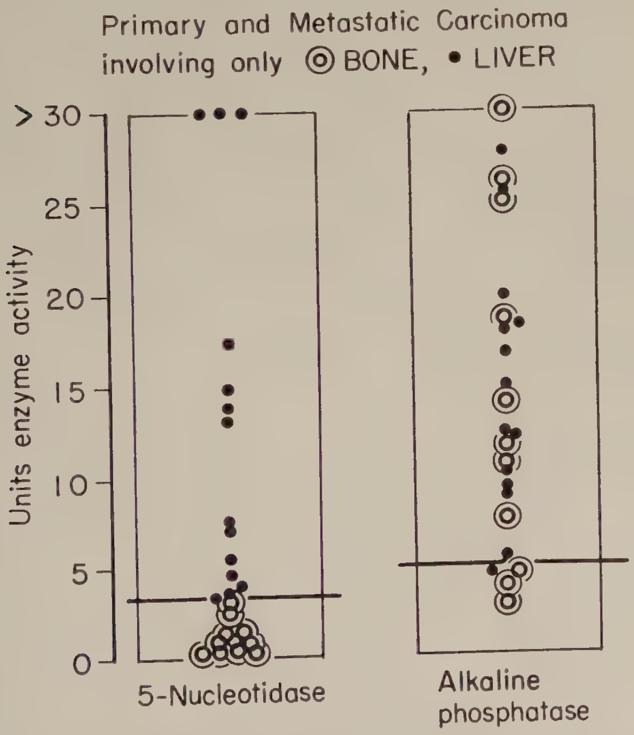

FIGURE 3. Serum phosphatase determinations in subjects with carcinoma of bone and liver.

can be distinguished from that due to neoplasm of the liver by the 5-nucleotidase determination.

Of considerable interest is the observation that 5-nucleotidase levels of normal children are in the adult range, in contrast to the higher alkaline phosphatase levels attributable to bone growth. Children with hepatocellular damage frequently will have alkaline phosphatase levels greater than 15 B. U. but, in 6 cases thus far studied, serum 5-nucleotidase levels have not risen above 8 units. In these cases the alkaline phosphatase ranged from 12.7 to 28 B. U. In one case of severe subacute hepatitis in an infant 5-nucleotidase values fell to zero, while the alkaline phosphatase persisted above 20 units. Further study of 5-nucleotidase in children will be necessary to assess its value in the differential diagnosis of jaundice.

The clinical observations summarized here suggest that the liver is the most likely source of serum 5-nucleotidase elevation in hepatobiliary disease, as may well be true of the serum alkaline phosphatase. However, no experimental studies have as yet been conducted to examine this hypothesis. Furthermore, the determination of serum 5-nucleotidase may prove to be of clinical value in view of the evidence that it is at least as sensitive as the serum alkaline phosphatase in detecting the presence of biliary tract obstruction, and is more selective because values are not increased in diseases of bone associated with increased osteoblastic activity.

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SERUM PEPTIDASES IN HEPATIC DISEASE

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Introduction

The number of peptidases occurring in the serum of healthy persons appears to be quite large.[1, 2] In acute hepatic necrosis the activity of some of these enzymes may be greatly increased, while the activity of others may either be increased somewhat or remain essentially unchanged (Fleisher, unpublished data). The present study concerns an investigation of two serum enzymes that are affected by hepatic disease; namely, aminotripeptidase and leucine aminopeptidase.

Methods

Peptidase activities were determined at 38°C. with an initial peptide concentration of 5×10^{-3} molar. For the aminotripeptidase,[2] the substrate was glycylglycylglycine and the buffer was Sörensen's phosphate, *p*H 6.6 and 7.7. No activating ion was added. The extent of hydrolysis after 1 and 2 hours was determined by means of the colorimetric ninhydrin method of Moore and Stein.[3] Tripeptidase activity was taken as the mean of the two determinations, calculated as initial reaction rates in μmoles of peptide hydrolyzed during 1 hour by 1 ml. of serum (μM/hour/ml.). For the leucine aminopeptidase the substrate was L-leucylglycine, the buffer was Michaelis' Veronal at *p*H 8.4, and the activating ion was manganese at a concentration of 2×10^{-3} molar. The method of analysis was similar to the tripeptidase procedure, the details of which have been described elsewhere.[4]

Results and Comment

Serum aminotripeptidase in hepatic disease. The hydrolysis of glycylglycylglycine by normal serum gave maximal rates at *p*H 6.8; in contrast, the sera of patients with hepatic disease showed not only an increase in hydrolytic rates, but also a shift of the optimal *p*H toward greater alkalinity (FIGURE 1). This different behavior suggested the existence of two serum components, namely tripeptidase A, with peak activity at about *p*H 6.8, which occurs preponderantly in normal blood, and tripeptidase B, with peak activity near *p*H 7.8, which predominates in blood during hepatic disease.

To investigate the significance of increased serum tripeptidase activity and of its *p*H shift in diseases of the liver, determinations were made simultaneously at *p*H 6.6 and *p*H 7.7. A group of 116 patients with various hepatic diseases, including also some with infectious mononucleosis, was compared with one of 39 healthy persons. Serial tests were performed on several of the 26 patients with viral hepatitis. TABLE 1 summarizes our results without regard to the

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* The Mayo Foundation, Rochester, Minn., is a part of the Graduate School of the University of Minnesota, Minneapolis, Minn.

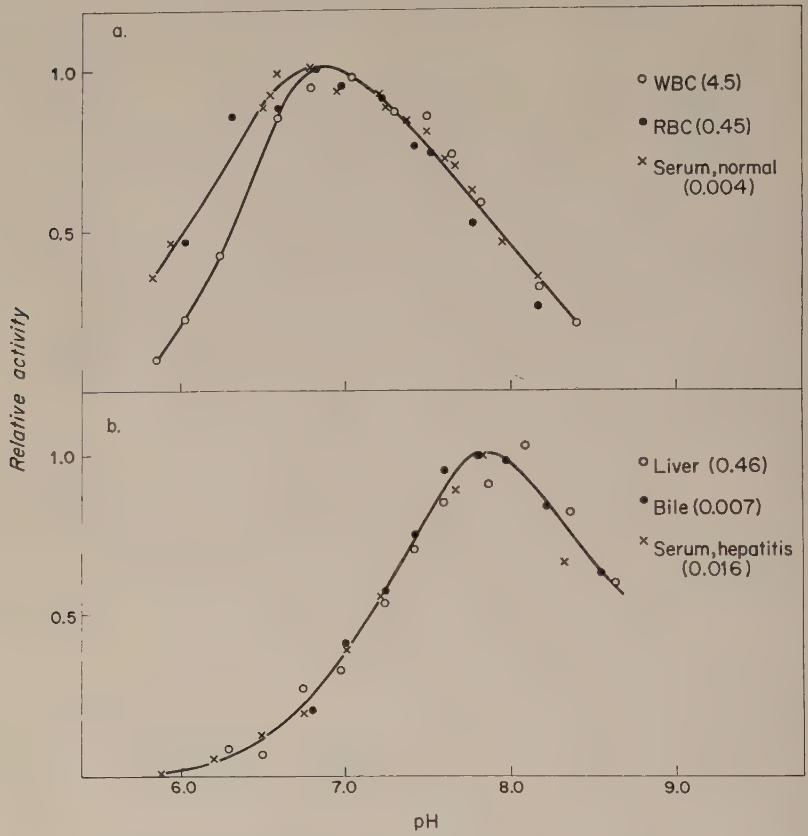

FIGURE 1. Effect of varying pH on the activity of serum aminotripeptidase. The broken line represents the difference of the two plots.

TABLE 1
COMPARISON OF AMINOTRIPEPTIDASE ACTIVITY BETWEEN NORMAL SERUM AND SERUM IN HEPATIC DISEASE

	Activity μM/hr./ml. of serum*		Activity quotient†
	pH 6.6	pH 7.7	
Normal (39 tests)	3.88 ± 0.25	3.03 ± 0.16	0.81 ± 0.02
Hepatic disease (148 tests)	6.79 ± 0.36	8.81 ± 0.38	1.62 ± 0.09
	$p < .01$	$p < .01$	$p < 0.01$

* The figures following the ± sign are standard errors of the mean. The p values refer to the t test.

† Activity quotient is the activity at pH 7.7 divided by the activity at pH 6.6.

TABLE 2

SERUM AMINOTRIPEPTIDASE ACTIVITY IN VARIOUS DISEASES OF THE LIVER

	Activity μM/hr./ml. of Serum*	
	*p*H 6.6	*p*H 7.7
Cirrhosis (29 tests)	4.09 ± 0.42 $p > .01$	5.89 ± 0.34 $p < .01$
Obstructive jaundice (32 tests)	7.00 ± 0.60 $p < .01$	8.95 ± 0.60 $p < .01$
Viral hepatitis (58 tests)	6.47 ± 0.47 $p < .01$	9.40 ± 0.45 $p < .01$
Neoplasm of liver (12 tests)	9.99 ± 2.24 $p < .01$	11.64 ± 2.96 $p < .01$
Infectious mononucleosis (17 tests)	9.83 ± 1.19 $p < .01$	9.49 ± 1.09 $p < .01$

* The figures following the ± sign are standard errors of the mean. Except for the group of viral hepatitis, one test was done on each patient. The p values refer to the t test.

type of hepatic lesion. The tripeptidase activity was significantly increased at both *p*H values, although the increase was proportionately greater at the alkaline *p*H. At *p*H 6.6, 39 per cent of the serum specimens had activity exceeding the normal mean plus 2 standard deviations, while at *p*H 7.7, 86 per cent fell outside the normal range. The shift in optimal activity toward greater alkalinity can best be appreciated by comparing the individual activity quotients, that is, the activity at *p*H 7.7 divided by the activity at *p*H 6.6. The mean activity quotient was 0.81 for the normal sera, but 1.62 for the pathological sera. The difference has statistical significance.

TABLE 2 shows a comparison of the various groups of hepatic diseases. The activities were least in cirrhosis, particularly at *p*H 6.6. The groups of obstructive jaundice and viral hepatitis test showed similar moderate increases in activity and comparable shifts of the optimal *p*H. In the rather small group of proved metastatic disease, one patient showed exceptionally high values; namely 39.6 and 44.3 μM/hour/ml., while the others had activities somewhat similar to the findings in obstructive jaundice and hepatitis. Among the patients with infectious mononucleosis, many had comparatively high activity at *p*H 6.6, thus resulting in a high incidence of normal activity quotients.

Comparison of aminotripeptidase of serum, liver, bile, and blood cells. Since it appeared likely that tripeptidase B has its origin in the liver, activity-*p*H curves were obtained from hepatic biopsies (FIGURE 2, bottom); these proved to be similar to the hypothetical curve that the serum tripeptidase activities had suggested (FIGURE 1). The serum from one case of infectious hepatitis was found to be well suited for a more direct comparison. In this patient the activity of serum tripeptidase B was greatly increased, while that of serum tripeptidase A was exceptionally low. When the activity-*p*H curves of this serum and of hepatic tissue were plotted relative to their respective maximal activities (FIGURE 2, bottom), they appeared to be indistinguishable. It may

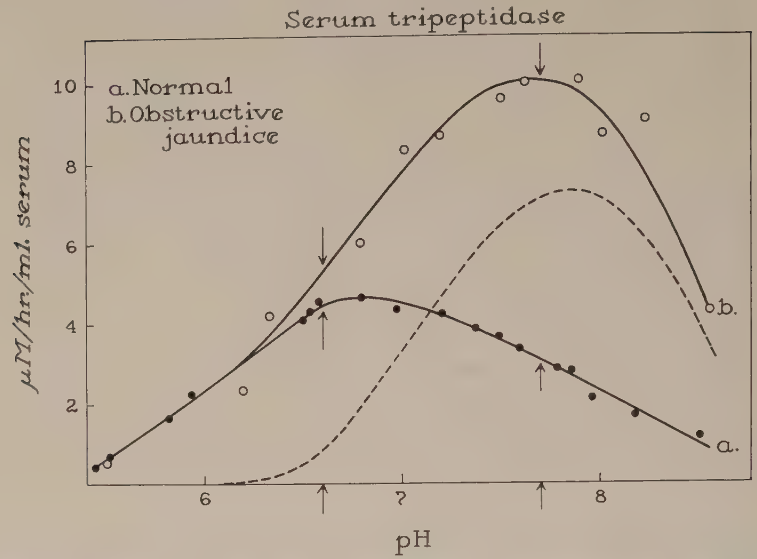

FIGURE 2. Effect of varying pH on the activity of aminotripeptidase from different sources. Figures in parentheses represent activities in μM/hour/mg. of wet weight under optimal conditions.

thus be concluded that the difference in serum tripeptidase between health and hepatic disease is due essentially to a difference in the source of the enzyme. Erythrocytes (Tsuboi *et al.*,[5] Brindley and Fleisher, unpublished data), lymphocytes and, particularly, polymorphonuclear leukocytes[6] contain highly active tripeptidase A (FIGURE 2, top). Considering the relatively short life span of these cells, the regular occurrence of this enzyme in normal serum is not surprising. Tripeptidase B, which is present in hepatic tissue in moderate amounts, appears to be cleared by way of the bile (FIGURE 2, bottom); in serum it is usually not detected except in the presence of hepatic cell damage or obstructive jaundice.

Serial determinations of serum aminotripeptidase activities in two patients with hepatitis. As was indicated before, patients with hepatic disease frequently show a simultaneous increase of both tripeptidases. This has been observed in approximately one of every three patients with either acute hepatitis or obstructive jaundice and in most patients with neoplastic disease of the liver. Serial determinations in two patients with acute hepatitis serve well to illustrate this difference in enzymatic patterns. Both showed the characteristic increase of tripeptidase B. One patient (FIGURE 3, top) also had a greatly increased activity of tripeptidase A in the initial stage of the disease, while the other (FIGURE 3, bottom) showed a consistently low activity at pH 6.6. The significance of this difference in enzymatic patterns in relation to the disease process has not been established. In this connection it is of interest to know that in infectious mononucleosis the situation is somewhat different. Our data indicate that an increase of tripeptidase A is the rule, and that tripeptidase B is also high in about one third of the patients.

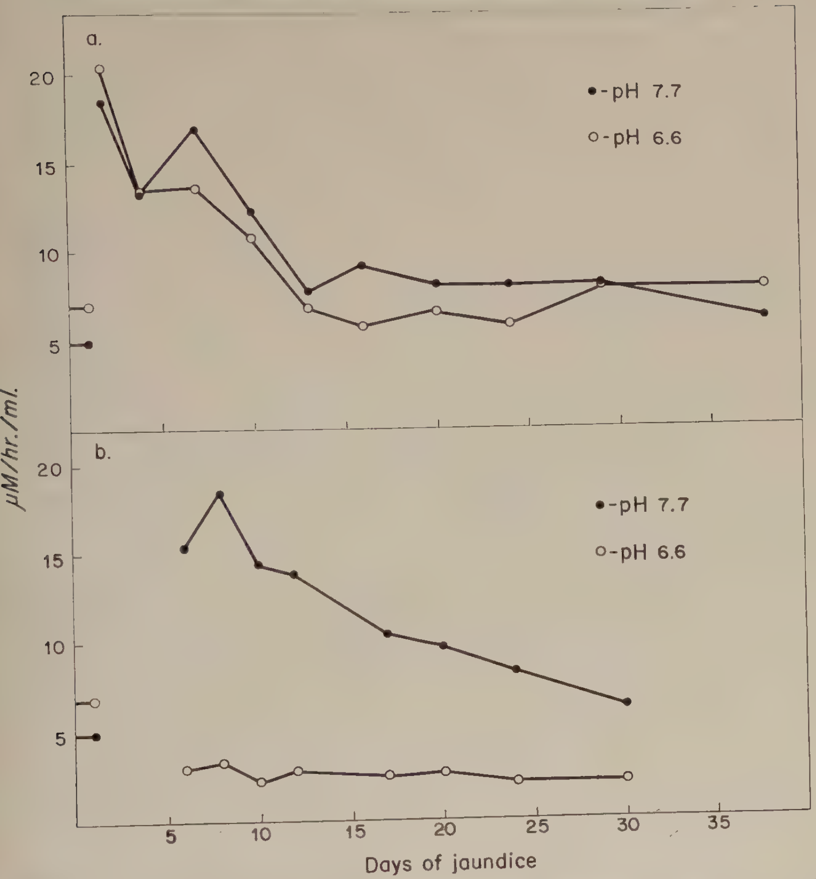

FIGURE 3. Serial determinations of aminotripeptidase in the sera of 2 patients with viral hepatitis. The activities indicated at the left margin are the normal means plus 2 standard deviations.

Aminotripeptidase in the dog. Our study was extended to include serum and hepatic tissue of the dog. As may be seen from FIGURE 4, significantly different activity-*p*H curves also were obtained in this species, although the two peaks were not as far removed from each other as in human beings. The activity of canine serum at its optimal *p*H of 7.2 is several times that of human serum, while the activity of hepatic tissue of the dog at its peak of *p*H 7.6 is similar to the activity of human liver.

Leucine aminopeptidase. The hydrolysis of L-leucylglycine is usually attributed to leucine aminopeptidase. There are, however, reasonable indications that normal serum contains other enzymatic components capable of splitting this dipeptide.[2, 7] The conditions of our test were designed to activate leucine aminopeptidase, but to retard other enzymes. The average rate in 82 normal persons was found to be 0.98 μM/hour/ml., a value considerably lower than the mean activity of tripeptidase A.

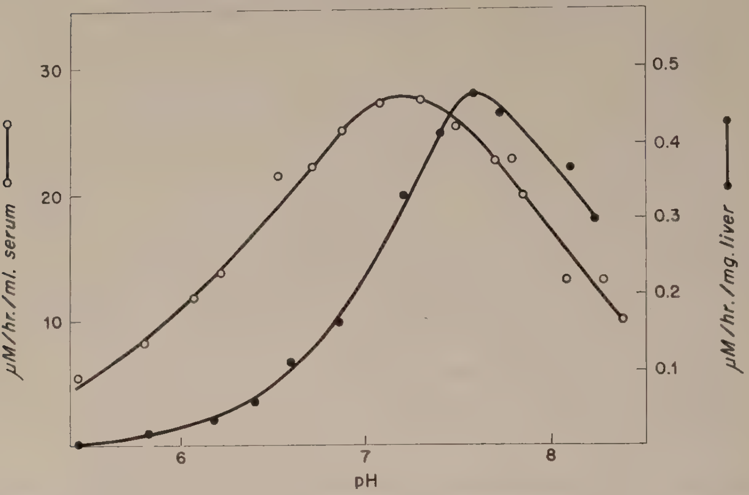

FIGURE 4. Effect of varying *p*H on the activity of aminotripeptidase in serum and liver of the dog.

Serum leucine aminopeptidase in hepatic disease. Our findings in 261 patients with hepatic disease and 41 patients with infectious mononucleosis are summarized in TABLE 3. When compared with the normal mean, a statistically significant increase was present in all types of disease. The difference was least in patients with cirrhosis and obstructive jaundice, namely 2.43 and 2.56 μM/hour/ml., respectively, and 48 and 65 per cent had activity exceeding the normal mean plus 2 standard deviations. The mean of the patients with neoplastic hepatic disease was greater; namely 4.42 μM/hour/ml., with 76 per cent falling outside the normal range. By far the greatest increase in enzymatic activity occurred in 32 patients with acute hepatitis, averaging 79.5 μM/hour/ml.

There was no difference between the patients with infectious hepatitis and those with homologous serum hepatitis. In this group a total of 99 individual tests was performed. When the results of these tests were arranged by weeks

TABLE 3
LEUCINE AMINOPEPTIDASE ACTIVITY IN SERUM

	Activity μM/hr./ml. of serum*
Normal (82 patients)	0.98 ± 0.04
Cirrhosis (81 patients)	2.43 ± 0.39
Obstructive jaundice (97 patients)	2.56 ± 0.21
Neoplasm of liver (51 patients)	4.42 ± 0.69
Viral hepatitis (32 patients)	79.50 ± 28.00
Infectious mononucleosis (41 patients)	35.20 ± 5.46

* The figures following the ± sign are standard errors of the mean.

TABLE 4

SERUM LEUCINE AMINOPEPTIDASE ACTIVITY IN HEPATITIS

Days after onset of jaundice	Tests	Patients	Activity	
			Mean	Range
			µM/hr./ml.	
0–7	24	13	100.0	2.6–755
8–14	27	18	30.5	1.3–141
15–21	16	12	16.9	1.9– 51
22–28	16	13	18.7	3.0–153
29–35	8	7	8.7	1.4– 23
36–56	8	6	2.6	1.9– 3.4

after the onset of jaundice (TABLE 4), it was noticed that the greatest changes occurred during the first week of the disease. There was not always a correlation between maximal enzyme activity and the severity of the disease, but it appeared that the time at which the peak occurred was important. Thus, some patients with mild disease had high enzymatic activity soon after the onset of jaundice, but showed a return to normal within a short time. In contrast, patients with severe disease usually had a delayed peak of activity, as did the one shown in FIGURE 5.

In patients with infectious mononucleosis, this peptidase activity also was greatly increased. The mean activity in 41 patients was 35.2 µM/hour/ml. with a range up to 206. Correlation between peptidase activity and liver function has not been apparent.

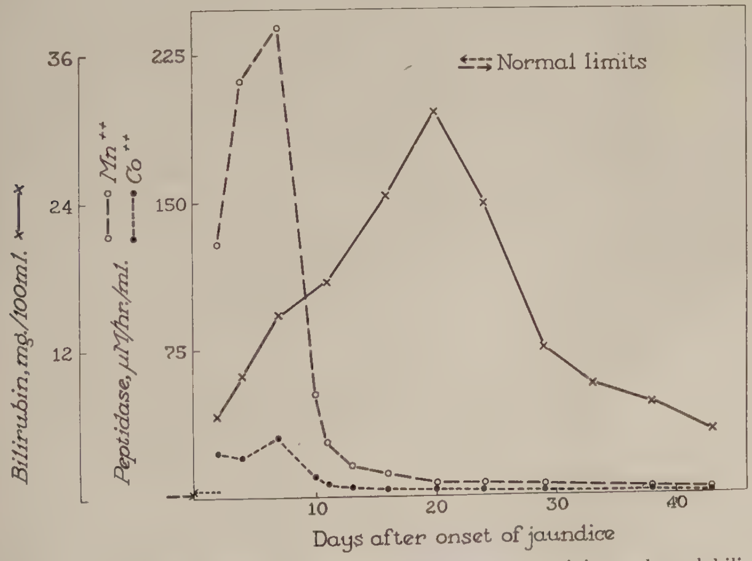

FIGURE 5. Serial determination of leucine aminopeptidase activity and total bilirubin in serum of a patient with homologous serum hepatitis.

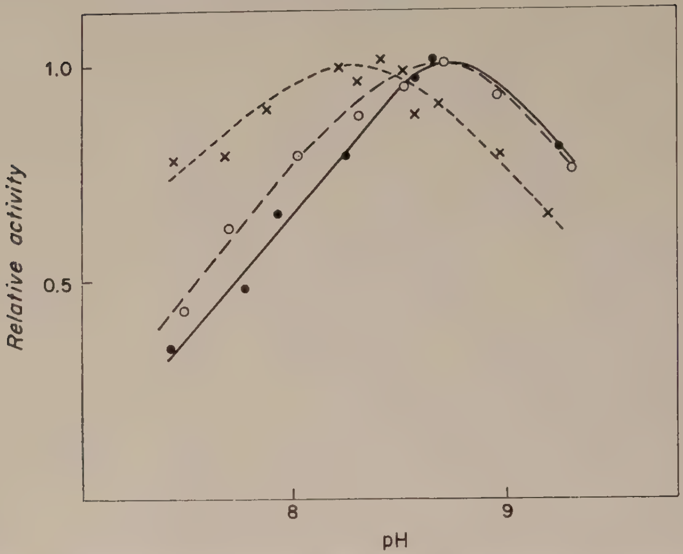

FIGURE 6. Effect of varying *p*H on the hydrolysis of L-leucylglycine in normal serum (×), serum in hepatitis (○), and liver tissue (●). Maximal activities were 1.05 and 50.6 μM/hour/ml., respectively, for the normal and hepatitis serum, and 9.4 μM/hour/mg. of fresh tissue for the liver.

Comparison of leucine aminopeptidase of serum and liver. Since the excessive serum peptidase activity in hepatic disease could be assumed to originate in the liver, a direct comparison of the *p*H-dependency between the enzymatic activity in hepatitis serum and that in hepatic tissue was made. As shown in FIGURE 6 the two activities had rather similar curves with maximums at *p*H 8.7 and 8.8. In contrast, the peak activity in several normal sera was about *p*H 8.3; this difference presumably can be attributed to the presence of one or more additional enzymes, which contribute more to the over-all activity of normal serum than of serum in hepatitis. Activities in the presence of cobalt instead of manganese have also been used to show the similarity between hepatitis serum and hepatic tissue. With both preparations, cobalt produced strong inhibition (this is shown for serum in FIGURE 5) while with normal serum this ion effected activation.[2]

TABLE 5

SERUM LEUCINE AMINOPEPTIDASE IN DOGS BEFORE AND 48 HOURS AFTER POISONING WITH CARBON TETRACHLORIDE

Dog	Activity	
	Before CCl_4	After CCl_4
	μM/hr./ml.	
1	2.2	85.0
2	2.1	147.0
3	2.0	99.5

Serum leucine aminopeptidase in experimental hepatic injury. The hydrolysis of L-leucylglycine by serum in experimental hepatic injury was studied in dogs following gastric intubation of carbon tetrachloride in doses of 2 ml./kg. of body weight. Maximal increases of peptidase activity up to 70 times the control values occurred 48 hours after severe poisoning (TABLE 5). Within the following 5 days, the activity declined rapidly to near normal. The relative peak activity in the dogs was less than in patients with hepatitis, presumably because of fourfold lower activity in liver and of twice greater serum activity in the dog as compared with human beings.

Summary

Two types of peptidase activity were studied in the serum of patients with hepatic disease, namely, aminotripeptidase acting on glycylglycylglycine, and leucine aminopeptidase acting on L-leucylglycine. Comparisons were made with the corresponding activities in normal serum, blood cells, hepatic tissue, and bile.

The study on aminotripeptidase indicates the existence of two different enzymes: tripeptidase A with optimal activity at about *p*H 6.8, which occurs predominantly in normal serum and in erythrocytes and leukocytes, and tripeptidase B, with optimal activity near *p*H 7.8, which is present in the liver and in bile. In the serum of patients with hepatic disease, tripeptidase A is usually accompanied by varying amounts of tripeptidase B, according to the results of simultaneous tripeptidase determinations at two values for *p*H, using 148 sera from 116 patients with hepatic disease.

Observations on the hydrolysis of L-leucylglycine likewise indicate the existence of more than one peptidase. However, this study is concerned only with the manganese-activated leucine aminopeptidase, which is present in normal serum in relatively low activity. Analysis of the serum from 261 patients with all types of hepatic disease revealed that this enzymatic activity is significantly increased in all groups. By far the greatest changes are observed in acute hepatitis, particularly during the early phase of the disease, when increases of more than one hundredfold may occur. Close similarities were noted between the activities of hepatitis in serum and in hepatic tissue, which suggests the liver as the source of the increased serum activity in this disease. Greatly increased activity of leucine aminopeptidase also was noted in the majority of 41 patients with infectious mononucleosis.

Acknowledgment

We are indebted to our colleague K. G. Wakim for his assistance in the studies of serum leucine aminopeptidase in experimental hepatic injury.

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IMMUNOCHEMICAL STUDIES ON ALKALINE PHOSPHATASE

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The long-range objective of the studies on the immunochemistry of enzymes is to define the tissue of origin of the enzymes in the serum. The enzymes discussed here are the alkaline phosphatases.

From the time the enzyme was first discovered in serum in 1924[1, 2] the question of the tissue of origin has interested many investigators. Based on different approaches to this problem, the main tissue of origin of serum alkaline phosphatase has been ascribed by different investigators to the intestines,[3, 4] liver,[5] and bone.[6] Statements that the enzymes from the various tissues are identical[7] are to be found in the literature, as are other statements to the effect that they are different.[8]

One cannot explain all of these discrepancies, but one of the reasons for these diverse claims comes from the use of different experimental techniques and approaches, and another from the fact that such studies involve the effects of activators and inhibitors on phosphatases of different purities.

The immunochemical approach was undertaken with the idea that, whereas the catalytic regions of the various phosphatases might be very similar, there might be other regions of the molecule in which structural differences would be more pronounced. If these regions were capable of acting as antigenic determinants, one could expect, upon immunization, to get antibodies capable of distinguishing among the phosphatases. With such antibodies it might then be possible to reinvestigate the question of the origin of the serum alkaline phosphatases.

Before considering the results that have been obtained on the differentiation of phosphatases of human origin I propose to discuss some of the characteristics of the phosphatase-antiphosphatase system as found in the dog. Portions of this work have already been described.[9–11]

Table 1 summarizes some of the enzymatic properties of the alkaline phosphatases prepared by a common procedure from three dog tissues (intestine, liver, and kidney). From the point of view of *p*H of optimum activity, activation by magnesium, and inhibition by fluoride, cyanide, and histidine at the particular levels used, there is a striking similarity between these enzymes. The data on the effect of taurocholate shows a wider separation of results but, as stated earlier, when dealing with impure materials (and these *are* impure materials) the interpretation of such differences is not free from ambiguity. While this is not an exhaustive study, it nevertheless emphasizes the similarity of the three phosphatase preparations.

Antisera against dog intestinal phosphatase (DIP) were prepared by injecting rabbits intravenously with alum-adsorbed suspensions of the enzyme. Table 2 shows the specificity of such antisera in the precipitation of the enzyme from several dog organs. It will be noted that the antibodies are specific for two different preparations of the intestinal phosphatase, precipitating them

TABLE 1

INFLUENCE OF pH, MAGNESIUM, CYANIDE, FLUORIDE, HISTIDINE, AND TAUROCHOLATE ON ACTIVITY OF ALKALINE PHOSPHATASES*

	DIP-A	DIP-B	DLP	DKP
Optimal pH	9.7	9.7	9.7	9.7
Activation by				
0.0125 M magnesium	1.3	1.4	1.8	2.4
0.0625 M magnesium			1.3	
Per cent inhibition by				
0.0125 M cyanide	99	99	98	97
0.01 M fluoride	4	6	15	16
0.01 M histidine	94	94	89	89
0.002 M histidine	64	69	78	60
0.01 M taurocholate	6	7	51	57
0.001 M taurocholate	4	3	12	26

* Data from Schlamowitz, reproduced by permission from *Journal of Biological Chemistry*.[10]

TABLE 2

PRECIPITATION OF ALKALINE PHOSPHATASES BY DOG INTESTINAL PHOSPHATASE ANTISERUM*

Enzyme	Precipitation by DIP-A antiserum	Precipitation of DIP-A from phosphatase mixture by DIP-A antiserum
	per cent	*per cent*
DIP-A	97–100	—
DIP-B	100	—
DLP	0	97
DKP	1	99

* Data from Schlamowitz, reproduced by permission from *Journal of Biological Chemistry*.[10]

completely, while leaving the enzymes from the liver or kidney untouched when tested alone or in a mixture with intestinal enzyme.

The catalytic site of the enzyme is not believed to be involved critically in this precipitation of enzyme by antibody, since suspensions of the enzyme-antibody precipitate show all of the activity of the free enzyme. Other evidence to support this point will come out in the discussion of the influence of cysteine on the system, which in turn follows a discussion of some of the quantitative aspects of the phosphatase-antiphosphatase system.

The quantitative aspects of this antigen-antibody system are shown in FIGURE 1. In the experiments that yielded these data, increasing amounts of antigen were added to a series of tubes containing a fixed amount of antiserum. After allowing a suitable period for precipitation to take place, the mixtures were centrifuged and the clear supernatant solutions analyzed for their phosphatase content. The percentage of enzyme activity remaining in the clear supernatant fluids is plotted against the amount of enzyme added initially. In control tubes, where normal serum is used instead of antiserum, no precipitation occurs, and 100 per cent of the activity remains in the supernatant. We note three regions of such a precipitin curve: (1) a region between 20 and 50 γ of antigen in which the antigen is completely precipitated by its antiserum; and (2) and (3) regions in antibody- and antigen-excess, respectively, where precipitation becomes less complete until the activity is all back in the supernatants.

The unprecipitated enzyme in the regions (2) and (3) is not free, but is present in the form of soluble complexes with antibody. This was demonstrated by the application of horse antirabbit γ-globulin antibodies to this system. Rabbit antibodies fall largely into the class of serum γ-globulins. It follows that if rabbit serum γ-globulins are used as antigens in another species (for example, the horse), there would be produced in that species an antiserum that will precipitate rabbit γ-globulins and, among these γ-globulins, the rabbit antibodies. Furthermore, it appeared likely that anything complexed to these rabbit antibodies, such as DIP complexed to antibody, would be carried down in such a precipitation of rabbit γ-globulin by its horse antibodies. Horse antirabbit γ-globulin serum was prepared; TABLE 3 shows the effects of its addition to systems in the regions of antibody and antigen excess where only partial precipitation of the enzyme by antibody ordinarily occurs. It will be noted that precipitation now is complete, confirming the existence of soluble complexes in both of these regions. In control tubes where normal serum is used in place of antiserum, horse antibodies have no influence on the enzyme. The use of these horse antibodies further provides a means of assuring complete precipitation of the antigen (phosphatase) when that enzyme is present in such low or in such high amounts as would give rise to soluble antigen-antibody complexes. This use of horse antirabbit γ-globulin to obtain complete precipitation of phosphatase by its antibody is stressed because of the use made of it in the

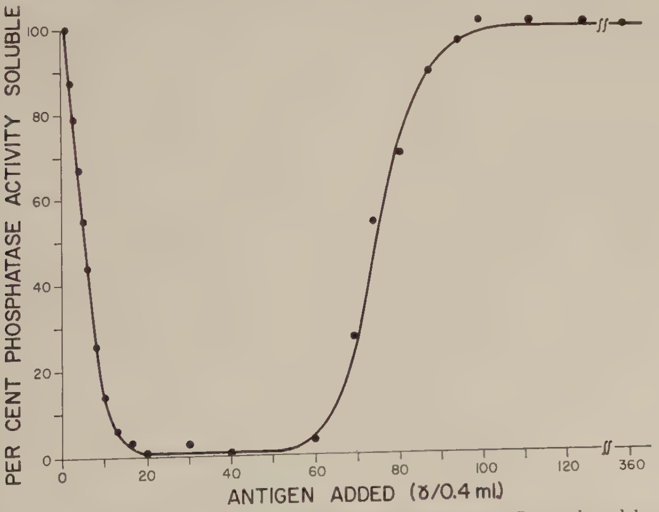

FIGURE 1. Quantitative aspects of this antibody system. Reproduced by permission from the *Journal of Immunology*.[11]

TABLE 3[11]

INFLUENCE OF HORSE ANTIRABBIT ANTIBODIES ON THE PRECIPITATION OF DIP BY ANTI-DIP SERUM*

DIP	Horse antirabbit antibodies	DIP activity precipitated
gamma		*per cent*
1	−	9
1	+	100
2	−	17
2	+	100
4	−	33
4	+	100
120	−	3
120	+	95

* Reproduced by permission from the *Journal of Immunology.*

studies of human sera to be described, where the amounts of enzyme are low and precipitation by rabbit antibody alone is incomplete.

Earlier it was mentioned that the catalytic site of the enzyme was not believed to be involved critically in the combination or precipitation of the enzyme by its antibody and that such evidence was derived from studies of the influence of cysteine on the system.

Treatment of the enzyme with cysteine destroys at least 97 per cent of its activity, but leaves intact its ability to combine with its antibody. To establish this latter point recourse was had to the quantitative precipitation characteristics of DIP with its antiserum described above. It will be recalled that at low levels of DIP (antibody excess), the system was in the range of soluble complexes, and that a similar region of soluble complexes obtained with high levels of antigen (antigen excess).

If the cysteine-treated, enzymatically inactive DIP has most of its antigenic determinant groups intact, then addition of it to low levels of normal DIP should carry the system out of the region of antibody excess to cause its complete precipitation. Similarly, the addition of large amounts of this "carrier" cysteine-treated DIP to normal DIP at levels where the DIP is quantitatively precipitated should cause the system to behave as in the region of antigen excess, with the DIP no longer precipitable. In other words, the retention of antibody-combining sites after cysteine treatment is being demonstrated by the use of cysteine-treated DIP as a "carrier."

TABLE 4 illustrates the data obtained from such experiments. One sees that DIP in levels not quantitatively precipitated by antiserum (1, 2, 4γ) are now, upon the addition of "carrier" (24γ), completely precipitated; and that amounts of DIP (37γ) completely precipitated under ordinary conditions are brought into a state of antigen excess and complete solubility by the addition of "carrier" (109γ). This evidence added to the previous kinetic data strongly supports the view that the antigenic and catalytic sites are not structurally identical.

With this background on the characteristics of phosphatase-antiphosphatase systems, studies on the differentiation of alkaline phosphatases from human tissues will now be considered.

TABLE 4

INFLUENCE OF CYSTEINE-TREATED DIP ON THE PRECIPITATION OF DIP BY ANTI-DIP SERUM

DIP	SH-DIP	DIP activity precipitated
gamma	*gamma*	*per cent*
1	—	9
1	24	100
2	—	17
2	24	100
4	—	33
4	24	98
37	—	94
37	109	4

TABLE 5

PRECIPITATION OF HUMAN INTESTINAL PHOSPHATASE (HIP) AND BONE ALKALINE PHOSPHATASE (HBP) BY RABBIT ANTIPHOSPHATASE SERA

Enzyme	Precipitation by	
	Anti-HIP serum	Anti-HBP serum
	per cent	*per cent*
HIP-EtOH*-II	90	1
HBP-A	0	92
HIP-EtOH-II + HBP-A	96 per cent of theory	98 per cent of theory

* A preparation of HIP prepared by fractionation from ethanol.

The phosphatases from human intestine and bone, and their respective antisera, have been prepared. TABLE 5 shows the results of the reaction of these enzymes with their antisera. It will be noted, first, that human intestinal phosphatase (HIP) is almost completely precipitated by its antiserum, but left untouched by the anti-human bone phosphatase (HBP) serum; second, that an enzymatically equivalent amount of HBP is almost completely precipitated by its antiserum, but left untouched by the anti-HIP serum; and, finally, that from a mixture of the two enzymes, the anti-HIP serum selectively precipitates out the HIP and the anti-HBP serum selectively precipitates out only the HBP.

Thus, under these conditions, the specificity of the antibodies for their antigens is noted, as is the absence of any cross reactions. It must be emphasized that the absence of cross reactions applies to the favorable conditions selected for these tests. Under conditions where the enzymes are present in very low levels as in serum and where horse antibodies are present, cross reactions of the anti-HBP serum with HIP may be observed, although the reverse, the cross reaction of anti-HIP serum with HBP, does not seem to occur.

Examination of the results on the differentiation of alkaline phosphatases in human serum shown in TABLE 6 indicates that, in the normal individual, ap-

TABLE 6

PRECIPITATION OF ALKALINE PHOSPHATASE FROM HUMAN SERA BY ANTI-HIP AND ANTI-HBP ANTISERA

Subject	Sex	Age	Clinical diagnosis	Serum phosphatase level	Phosphatase activity precipitated by		
					Anti-HIP	Anti-HBP	Anti-HIP + Anti-HBP
				K. A. units	*per cent*	*per cent*	*per cent*
M.S.	M	38	Normal	8.4	28	67	69
J.W.	F	52	Ca. breast, liver, & skeletal metastases	118.0	8	92	91
G.H.	M	71	Ca. cystic duct obstructive jaundice	61.8	3	80	78
C.S.	M	45	Ca. kidney (removed) liver metastases	49.8	11	87	86

proximately 27 per cent of the serum phosphatase is attributable to the intestine, as deduced from the amount precipitated by anti-intestinal antibody in the presense of horse antibodies. The percentage contribution by intestine in the case of patients with elevated serum phosphatases was much less, although the absolute contribution in two of the cases was greater than normal.

The percentage of total serum phosphatase precipitated by anti-HBP serum was 67 per cent for the normal and from 80 to 92 per cent for the pathological cases. A glance at the last column of TABLE 6 makes it clear that the anti-HBP serum under these conditions has cross-reacted and precipitated the HIP, and that all of this enzyme cannot be attributed to bone origin. At best, a subtraction of the intestinal contribution leaves 41, 83, 75, and 75 per cent, respectively, the contribution by bone for the 4 cases; this is still by far the major contribution to the serum phosphatase. However, in view of the present lack of information on possible cross reaction of anti-HBP with phosphatases from tissues other than intestine or bone, these figures should for the present be termed nonintestinal serum phosphatase.*

Much more data from normal and pathological sera will be required to determine whether in cases of elevated serum phosphatase there is a disturbed pattern in tissue contributions of the enzymes or simply an accumulation of the enzymes in their normally existing ratios.

Because the results of only four cases are available at present and because the quantitative and qualitative contributions of phosphatases from other tissues (liver, kidney, and spleen) have not yet been ascertained, we defer any firm conclusion concerning the tissues of origin of the serum alkaline phosphatases and look upon the data as an indication that this objective may be attainable.

* Additional experiments have shown that human liver and kidney phosphatases are precipitated by anti-HBP serum in the presence of horse antibodies, and that they are not affected by anti-HIP serum. It remains to be established whether the enzymes from these two sources contribute significantly to the serum phosphatase.

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Part V. Clinical Significance of Blood Enzymes

SUMMARY OF PANEL DISCUSSION ON CLINICAL SIGNIFICANCE OF ENZYMES IN BLOOD

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Herein is summarized the panel discussion that concluded the conference upon which this monograph is based. The aims of the panel were, first, to give brief expression to some views of the clinical applications of the subject that the various members of the panel had not had an opportunity to present in the regular sessions; second, to answer in free discussion questions that had arisen in the minds of participants; and, finally, to summarize briefly the general aspects of the application of blood enzyme determinations to clinical medicine, both as regards its present status and future outlook. The author was chairman; the other members of the panel were William S. Beck, Massachusetts General Hospital, Boston, Mass.; William Fishman, Tufts University Medical School, Boston, Mass.; Alexander Gutman, College of Physicians and Surgeons, Columbia University, and Mount Sinai Hospital, New York, N. Y.; Paul A. Marks, Francis Delafield Hospital, New York, N. Y.; I. H. Scheinberg, Albert Einstein Medical College, Yeshiva University, New York, N. Y.; Laurens P. White, Stanford University School of Medicine, San Francisco, Calif.; and Felix Wróblewski, Sloan-Kettering Institute for Cancer Research and Memorial Hospital. The present report is in no way comprehensive; it merely indicates the main points that were considered.

William Fishman began the discussion by asking me to define the "normal value" of a serum enzyme activity or, even more broadly, of any biochemical component of blood. I replied that, as is generally appreciated, a numerical measure of the probability that a given value is abnormal may be obtained by noting by how many standard deviations this value is lower or higher than the mean value for a group of normal individuals. For example, a value that is higher than the mean by two standard deviations is considered "abnormal" in the sense that such a value would occur only about twice in one hundred normal persons. However, the exact number of standard deviations to be used as a criterion might differ for different types of determinations and different clinical conditions, depending upon the gravity of the decision to be made. It is conceivable, for example, that a determination that is repeatedly different from the normal mean by one and one-half standard deviations may be crucial in the diagnosis or management of a serious disease.

One participant disagreed with this view and expressed the opinion that the value for a serum enzyme activity that might be normal for one individual might be abnormal for another, and that it was preferable to establish the norm for each individual before making a judgment as to what constituted an abnormal deviation. It was readily agreed that knowledge of the intraindividual variation is indeed helpful, but it was pointed out that this could be achieved only in special, closely controlled investigative situations in which a patient could be observed for long periods. This is not possible in the usual clinical situation where diagnosis and management must be accomplished as readily as possible.

Since the subject of metalloenzymes and metalloproteins in serum had received limited attention during the conference, I. H. Scheinberg was asked to summarize this field. Scheinberg answered that he would confine his discussion to ceruloplasmin. The normal concentration of this copper-containing, blue alpha-globulin in plasma is about 30 mg. per 100 cc.; it thus constitutes about 0.5 per cent of the plasma proteins. The concentration of ceruloplasmin is decreased in patients with hepatolenticular degeneration (Wilson's disease). In a series of 26 such patients, the concentrations of ceruloplasmin ranged from 0 to 14 with an average of 5 mg. per 100 cc. Other investigators have also found marked decreases of ceruloplasmin in this disease. This component is also decreased in the nephrotic syndrome and transiently in the newborn infant, and is increased above normal in pregnancy and several diseases. However, it would appear that in Wilson's disease the deficiency or absence of ceruloplasmin in the serum is due to a specific inherited inability to synthesize this protein at a normal rate.

Since patients with Wilson's disease show an excessive net absorption of copper, as well as a deficiency of ceruloplasmin, several investigators have suggested that the physiological role of this protein is to diminish copper absorption and/or to promote its excretion. Such a role might be mediated by reversible release and binding of copper by ceruloplasmin, phenomena that have been demonstrated *in vitro*. These considerations, and the fact that no clinical or biochemical abnormalities other than those connected with copper metabolism have been demonstrated in infants lacking ceruloplasmin, raise the possibility that the oxidase activity of ceruloplasmin may have no physiological significance.

William Fishman was then asked to discuss his work on serum prostatic acid phosphatase. Fishman noted that this determination was based on the inhibition of prostatic acid phosphatase by tartaric acid. Consequently, the difference between serum acid phosphatase activities in the presence and absence of tartaric acid provides a measure of that portion of the serum acid phosphatase that comes from the prostate gland. Fishman reviewd his work on approximately 100 cases of carcinoma of the prostate and noted that, when the conventional determination was employed, elevated serum acid phosphatase values were obtained in about 35 per cent of all cases of carcinoma of the prostate, 47 per cent of those with bony metastases, 16 per cent of those with soft tissue metastases, and about 15 per cent in those with no metastases. In contrast, Fishman noted, the use of the determination of prostatic acid phosphatase yielded a much higher incidence of elevated values, namely, about 83 per cent in all cases of prostatic carcinoma, 85 per cent in those with bony metastases, and 80 per cent in those with no metastases.

At this point Alexander Gutman observed that Fishman's values of about 41 per cent for the incidence of elevated values for "total" serum acid phosphatase in metastatic prostatic carcinoma appeared low. In his original studies in 1937 Gutman had observed that the conventional serum acid phosphatase activity (that is, the total acid phosphatase as referred to by Fishman) was elevated in about 80 per cent of cases with bony metastases; that at that time other investigators had obtained similar results; and that such incidences were still being obtained in untreated cases. It would not appear, therefore, that the determination of the prostatic acid phosphatase would offer additional diagnostic aid in this group of cases. With regard to the use of the prostatic acid phosphatase determinations in patients without metastases, Gutman stated that he had had only limited experience, but that he had not observed that significant diagnostic aid was obtained by the use of the determination of the prostatic acid phosphatase.

Gutman also commented on the interesting finding of Lester R. Tuchman and his associates at Mount Sinai Hospital, New York, N. Y., that the serum acid phosphatase is elevated in patients with Gaucher's disease. All of twelve successive patients have thus far exhibited these increased values, which are about double or triple the upper limit of normal when phenylphosphate is used as substrate. Minimal elevations are obtained when sodium beta-glycerophosphate is employed as substrate. Tartrate does not inhibit this increased moiety of serum acid phosphatase.

At this point, H. M. Lemon, Boston University Medical School, Boston, Mass., commented on his findings with copper-resistant acid phosphatase. When phenylphosphate is used as substrate, Cu^{++} ion inhibits erythrocyte acid phosphatase completely, but inhibits the acid phosphatase activity of other tissue extracts negligibly, perhaps only to the extent that they are mixed with erythrocyte phosphatase. Lemon and his associates have observed that the serum copper-resistant acid phosphatase is elevated, not only in males with prostatic carcinoma (72 per cent), but also in about 20 per cent of male and female patients with nonneoplastic disease, 31 per cent of females with nonmammary metastatic carcinoma, and 74 per cent of females with mammary metastatic carcinoma.

The panel discussion then considered problems of more general interest. Felix Wróblewski noted that in the past few years the study of serum enzymes had provided diagnostic aid to clinical medicine. He raised the stimulating question dealing with the extent to which developments in the field of blood enzymes might contribute further to the problems of diagnosis and management in clinical medicine.

Laurens P. White was called upon to comment on this subject, since the latter had studied the concomitant alterations of several enzyme activities in myocardial infarction and other diseases. White pointed out that many enzymes are widely distributed in the tissues of the body. It might be expected that necrosis of tissues such as the myocardium might not be different from necrosis of skeletal muscle, liver, kidney, or brain. Any cause of tissue injury, whether it be infection, trauma, infarction or carcinoma, and whether or not it produces death of the tissue, may conceivably be associated with abnormal elevation of serum enzyme levels. For example, Laurens White has found that elevations of serum glutamic-oxalacetic transaminase, lactic dehydrogenase, aldolase and phosphohexose isomerase were frequently found in patients with myocardial infarction, and in a variety of other diseases as well, notably metastatic carcinoma. He was unable to obtain close correlation of the serum enzyme abnormality with the activity of the various enzymes in tissue homogenates. Thus patients with myocardial infarction do not demonstrate abnormal serum levels of isocitric dehydrogenase, but myocardium is rich in this enzyme. Such discrepant findings force a reconsideration of the many factors involved in the production of abnormal serum levels of many enzymes.

The chairman agreed with much of what Laurens White said, but noted that the serum enzymes that have thus far found diagnostic appplication can be divided broadly into two groups. The first includes those with a fairly specific relationship to a tissue of origin and that possess a fairly high degree of specificity with regard to a particular disease or group of diseases; serum alkaline phosphatase, serum acid phosphatase, and serum amylase are enzymes of this type. The enzymes of the second group are involved in important intermediary metabolic sequences and are thus found in practically all tissues, although they may vary in concentration or activity from tissue to tissue. Phosphohexose isomerase, phosphoglucomutase, lactic dehydrogenase, transaminase, and isocitric dehydrogenase are examples of this second group. As White had noted, and as several other investigators also had reported, damage or necrosis of a tissue is, therefore, likely to lead to the passage of many of these metabolic enzymes into the circulation. This does not mean that these enzymes will all be elevated to the same extent in damage to a particular organ, or that the same enzyme will be elevated in damage to different organs.

The degree of elevation of an enzyme in the circulation is probably dependent upon several factors: the concentration of the enzyme in the tissue, the rate of release of the enzyme protein from a physiologically or anatomically deranged tissue, and the rate of or removal from the circulation, whether by excretion or metabolism. It is thus possible that the pattern and time sequence of the elevation of one of these enzymes after damage to a particular tissue may be fairly distinctive and thus of aid in diagnosis. Certainly, transaminase has proved to be of value in this connection in following patients with myocardial infarction or infectious hepatitis. The possibility also exists that the still-accelerating search for serum enzymes will result in the discovery of some that are highly specific for damage to a particular tissue.

Within recent years many diseases listed as inborn errors of metabolism, such as phenylpyruvic oligophrenia or Von Gierke's disease, have been shown to be due to enzymic deficiencies, and these deficiencies are demonstrable in the liver taken at autopsy or biopsy. Kirkman and Kalckar, in their presentation, showed that the deficiency of the enzyme phosphogalactose uridyl transferase, which characterizes galactosemia, may be demonstrated, not only in the galactosemic liver, but also in the erythrocyte. Since the erythrocytes are readily available biological material, Paul Marks of the Francis Delafield Hospital, New York, N. Y. was asked to comment on the possibility that further investigation of the enzyme systems of the erythrocytes might result in the development of techniques of value in the study of diseases with enzyme defects.

Marks replied that the studies that he and Ruth T. Gross had reported on drug-induced hemolytic anemias earlier in this conference would fall into this class. As previous investigators had shown, the primaquine-sensitive subject shows a deficiency of glucose-6-phosphate dehydrogenase in the erythrocyte. In addition, Gross had observed deficient activity of this enzyme in the erythrocytes of subjects who had recovered from hemolytic anemias induced by fava beans and by the ingestion of napthalene. That this phenomenon has a genetic basis is demonstrated by studying the incidence of reduced erythrocyte glucose-6-phosphate dehydrogenase activity in subjects with histories of drug-induced hemolytic anemias, healthy subjects in a control population, and healthy relatives of persons in the first two groups. Marks noted that at present he knew of no other disease states that are characterized by alteration of the erythrocyte enzyme pattern, and he could not predict whether further studies would reveal such alterations.

The extent to which the enzyme activities of leukocytes, particularly alkaline phosphatase, had already found diagnostic application in the study of leukemias was briefly noted by William S. Beck. It was also pointed out that detailed papers had been presented during the conference on the respiratory, glycolytic, and phosphogluconate oxidation enzyme systems and on the nucleic acid metabolism of normal and leukemic leukocytes. That such studies could contribute to a knowledge of metabolic derangements not only in the leukemias but also in other types of neoplasia was recognized. It was noted that Lowy and his associates found that the concentration of ascorbic acid in the white blood cell-platelet layer was an indicator of the total ascorbic acid content of the body. The possibility was raised that further studies of the white cells of man might show them to be reflectors or indicators of metabolic processes in other generally inaccessible tissues of the body.

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